

AUSTRALIAN JOURNAL
OF
BIOLOGICAL SCIENCES

VOLUME 10

MELBOURNE
1957

AUSTRALIAN JOURNAL OF BIOLOGICAL SCIENCES

A medium for the publication of results of original scientific research in the biological sciences with special emphasis on the experimental phases. Volumes 1 to 5 issued as the Australian Journal of Scientific Research, Series B: Biological Sciences.

Published by the Commonwealth Scientific and Industrial Research Organization.
Issued quarterly, £2 per annum.

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Australian Journal of Biological Sciences,

Commonwealth Scientific and Industrial Research Organization,

314 Albert Street, East Melbourne, C.2, Victoria

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SYMBIOTIC VARIATION IN *TRIFOLIUM AMBIGUUM* M. BIEB. WITH SPECIAL REFERENCE TO THE NATURE OF RESISTANCE

By F. W. HELY*

[Manuscript received July 9, 1956]

Summary

(i) *Trifolium ambiguum* M. Bieb. was differentiated into a polyploid series of $2n$, $4n$, and $6n$ races, which were examined in symbiosis with a number of strains of nodule bacteria.

(ii) Sixty-four isolates of *Rhizobium trifolii* obtained from *T. ambiguum* growing in Australia and New Zealand failed to nodulate the majority of tube-grown plants of this species. This marked resistance to nodulation was also shown by very sparse and greatly delayed nodulation on the occasional susceptible plant.

(iii) These isolates, with a single exception which was slightly effective, were wholly ineffective on *T. ambiguum*; they were mostly effective on *Trifolium repens* L. and much less effective on *Trifolium subterraneum* L.

(iv) Strains of *Rh. trifolii* originally obtained from Turkey (within the natural range of *T. ambiguum*) nodulated this species more readily. The different races showed marked variation in susceptibility and response. The few "resistant" plants appeared to belong to that part of the host population which became susceptible to infection only at a late stage in growth.

(v) The effectiveness of these strains on *T. ambiguum* ranged from fairly effective to ineffective (parasitic); they were ineffective on both *T. repens* and *T. subterraneum*.

(vi) Even with the most effective symbiosis observed, the nodule number was low and the total volume of nodular tissue comparatively small.

(vii) A larger number of infections was found with increase in chromosome complement in *T. ambiguum*, in contrast to observations made on *Trifolium pratense* L., *T. subterraneum*, and *Medicago tribuloides* Desr.

I. INTRODUCTION

Trifolium ambiguum M. Bieb. occurs naturally in the Caucasus region where it is utilized for hay and as a perennial pasture legume (Larin 1937; Bobrov 1950). The desirable agronomic properties of this clover include a rhizomatous habit and great root storage capacity, drought hardiness, tolerance of waterlogged conditions, palatability, and high honey production.

The establishment of this species in agriculture outside this region has failed primarily because of the difficulty which has been experienced in obtaining good nodulation. In European and American trials nodules appear to have been wholly absent or sparse and entirely ineffective in benefitting the host by nitrogen fixation (Keim 1954). No mention appears to have been made in the Russian literature of any imperfections in its symbiosis. Hely, Bonnier, and Manil (1953) investigated the resistance of this species to infection. They were able to demonstrate that it could be overcome by grafting with a scion of *Trifolium repens* L. and that the nodules so produced were small and ineffective. Isolates obtained from such nodules did not

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become more compatible with *T. ambiguum* when passaged a number of times through grafted plants (Hely, Bonnier, and Manil, unpublished data). A solution to the problem has also been sought by Guravich (1949) and Keim (1953) in experiments on interspecific hybridization with a view to breeding for susceptibility and symbiotic effectiveness. They found that hybrid embryos resulting from crosses between *T. ambiguum* and *Trifolium hybridum* L. and other species did not persist unless cultivated on artificial media. Such embryos grown into mature plants did not have the desired nodulating ability and all plants which flowered were characterized by both male and female sterility, so that this approach to the problem had therefore to be discontinued.

An intensive and systematic study has been undertaken by Parker and Allen (1952) on the nodulation of *T. ambiguum* with isolates of bacteria taken from the occasional small nodules produced on *T. ambiguum* grown in Wisconsin and with strains of nodule bacteria isolated from a number of other species of *Trifolium*. None of these strains was observed to give any degree of effectiveness in symbiosis with *T. ambiguum*; moreover, the majority of strains failed even to nodulate this species.

Recently Erdman and Means (1954, 1955) have obtained bacterial strains from eastern Turkey (isolated from nodules on *Trifolium ochroleucum* Huds. and *Trifolium spadiceum* L. and from soil in which *T. ambiguum* occurred). These regularly nodulated *T. ambiguum* and some were shown to be effective on this host in pure culture.

The object of this report is to re-examine strain and host plant differences in the symbiosis of *T. ambiguum*, utilizing a wider range of plant material than has been employed hitherto. Particular emphasis will be placed upon host variation and its bearing upon the problem of the resistance of this species to infection. Another publication will deal with nodule anatomy and cytochemistry.

II. MATERIALS AND METHODS

(a) Plant Material

The source of each introduction of *T. ambiguum* employed in this investigation is given in Table 1 together with notes on growth and habit as observed in field plots at Canberra. The seed samples of the first six introductions listed came directly from Russian sources and were not known to be special selections, although it is clear from Table 1 that they vary in important agronomic characteristics.

The chromosome number of *T. ambiguum* was originally determined by Karpechenko (1925) as $2n = 16$, and was so quoted by Senn (1938). However, Trimble (1951) and Keim (1953) each reported a somatic number of 48. To clarify the situation chromosome counts were carried out for all introductions on root tips and to a less extent on pollen mother cells. These are given in Table 1 and it is patent that the species constitutes a polyploid series of diploids, tetraploids, and hexaploids with a basic $2n$ number of 16.

Seed was produced in isolated plots of original plants with pollinations carried out by hand or by honey bees. Apparently the plants of all samples were almost wholly self-incompatible, with less than 1 per cent. self-fertility, and they were

evidently highly heterozygous. With the exception of the introductions numbered $2n(1)$ and $6n(3)$ flowering was infrequent in Canberra and very little seed was obtained. For this reason intensive nodulation studies could only be carried out with the strains $2n(1)$, $6n(1)$, $6n(3)$, and $6n(7)$.

TABLE 1
PLANT MATERIAL USED IN THESE INVESTIGATIONS

Identification	Country from which Received	Symbol Used in Text†	General Description of Introductions as Grown in Canberra, A.C.T.			
			Plant Size	Type of Growth	General Vigour	Flowering and Seed Production
C.P.I.2264*	U.S.S.R.	$2n(1)$	Small	Dense, leafy, fine	Fair	Good
C.P.I.2771	U.S.S.R.	$2n(2)$	Small	Dense, leafy, fine	Fair	Fair
C.P.I.6884	U.S.S.R.	$4n(1)$	Small to medium	Open, stemmy, coarse	Poor	Erratic
C.P.I.9949	U.S.S.R.	$4n(2)$	Medium to large	Dense, leafy, moderately fine	Fair	Erratic
C.P.I.6161	U.S.S.R.	$6n(1)$	Large	Moderately dense, leafy, and fine	Good	Poor
C.P.I.10803	U.S.S.R.	$6n(2)$	Large	Moderately open and stemmy, fairly fine	Fair	Poor
C.P.I.18115	New Zealand	$6n(3)$	Large	Open, moderately stemmy, coarse	Poor	Erratic
C.P.I.20871	England	$6n(4)$	Large	Moderately open and leafy, coarse	Fair	Poor
F.C.23461†	U.S.A.	$6n(5)$	Large	Open, moderately stemmy, coarse	Fair	Poor
P.I.108699-C-39†	U.S.A.	$6n(6)$	Large	Moderately open, leafy, coarse	Good	Erratic
P.Q.-X45919†	U.S.A.	$6n(7)$	Large	Moderately open and leafy, coarse	Fair	Poor

* Commonwealth Plant Introduction number.

† United States Department of Agriculture number.

‡ $2n = 16$.

(b) Bacterial Strains

Because of the known inability of most strains of *Rhizobium trifolii* to nodulate this species, *T. ambiguum* itself was utilized as the selecting agent for strains. This was done by planting all available strains of *T. ambiguum* in composite soil consisting of turf taken from clover pastures (mainly *T. repens*) collected at different places in south-eastern Australia. Isolations were made later from the occasional nodules which were found. Diploid, tetraploid, and hexaploid introductions were used in this way to isolate a total of 60 strains. Nodules on the collecting plants were

variable in number and size but, generally, were infrequent, small or very small, and white in colour. They occurred scattered on the finer roots and were fugacious. In addition, four isolations originating in Asia Minor were kindly provided by Dr. Erdman, United States Department of Agriculture, and four isolations from nodules on plants of *T. ambiguum* grown at Palmerston North, N.Z., in a *T. repens* environment, were kindly made available by Mr. Greenwood, Department of Scientific and Industrial Research, N.Z.; these are listed in Table 2 with further details of their origin.

TABLE 2
STRAINS OF RHIZOBIUM TRIFOLII FROM EASTERN TURKEY AND NEW ZEALAND

Symbol Used in Text	Sender's No.	Origin
A	3Dly8	Isolated by Dr. L. W. Erdman at Beltsville, U.S.A., from nodule on <i>Trifolium ambiguum</i> grown in U.S.A. on soil taken from around roots of plant of this species growing naturally in eastern Turkey
B	3Dly8(c)	Same as above
C	3Dly15	As above but soil from another location in same region
D	3Dly19(c)	Isolated by Dr. L. W. Erdman from nodule on <i>Trifolium ochroleucum</i> growing naturally in eastern Turkey
E	1	Isolated by Mr. R. M. Greenwood at Palmerston North, N.Z., from sparsely occurring ineffective nodules found on hexaploid plants of <i>T. ambiguum</i> growing in the field
F	1a	Same as above
G	2	Same as above
H	3	Same as above

(c) Cultural Methods

Tests of symbiosis were carried out under bacteriologically controlled conditions as described by Thornton (1930). Surface-sterilized seed was sown in test tubes on agar slopes of a mineral salts medium lacking combined nitrogen. After germination the cultures were transferred to a louvre-shaded glass-house. Full shade was provided for the roots by standing the tubes upright in solid wooden racks. Watering was carried out with seedling-salts solution under sterile conditions.

Observations were made on nodulation and growth during the course of each experiment and, at harvest, dry weights, nodule number, and nodule size were determined.

III. EXPERIMENTAL RESULTS

(a) *Nodulation of T. ambiguum by Australian Isolates*

The response of $2n$ and $6n$ introductions of *T. ambiguum* to inoculation by Australian strains isolated from $2n$, $4n$, and $6n$ collecting host plants, as described above, is given in Table 3. Four replicated agar plant cultures of each kind were inoculated with each strain and as the object of this test was exploratory, i.e. to detect strains of good nodulating capacity and some degree of effectiveness, the results were taken only in terms of the average strain effect. This obscures much of the host resistance since the bacterial strains classified as ineffective or partially effective generally nodulated only one or two of the four inoculated plants. With this in mind it is evident that both diploid and hexaploid *T. ambiguum* lines are largely resistant to the Australian strains, the diploid possibly being slightly less resistant than the hexaploid. There is also a suggestion that the isolates from diploid collecting hosts may be less infective than the others.

The results also show that only one strain of the 60 examined exhibited any degree of effectiveness. This strain was very slightly effective on the $2n(1)$ host and moderately effective on the $6n(3)$ host. The occurrence of effectiveness with this isolate on both kinds of plant material indicated that a true bacterial strain difference was concerned. This was confirmed in a subsequent experiment, in which two of the $2n(1)$ plants and three of the $6n(3)$ plants gave slightly effective responses out of totals of 17 and 28 plants examined.

The effectiveness of these strains was also determined for *Trifolium subterraneum* L. and *T. repens* (Table 3). Nearly all were effective or partially effective on white clover and ineffective on subterranean clover.

The New Zealand isolates behaved similarly to the Australian; they were either ineffective or did not nodulate *T. ambiguum* and were largely effective on white clover; three of the four strains were wholly ineffective on subterranean clover.

These results therefore confirm those of Parker and Allen (1952) who found that isolates effective on white clover and ineffective on subterranean clover did not form nodules or were ineffective on *T. ambiguum*.

(b) *Nodulation by Turkish Strains of Rh. trifolii*

(i) *Experiment 1*.—As with the Australian isolates the first test was exploratory and employed varying numbers of replicates depending upon the amount of seed then available. The test plants were grown in agar culture and those which formed nodules were graded for effectiveness at 80 days after inoculation. The results in Table 4 give the number of plants without nodules (as a percentage of the total) and the proportions which responded ineffectively, intermediately, or effectively (expressed as percentages of those nodulating). The Turkish strains nodulated all host lines much more readily and effectively than any of the Australian or New Zealand isolates. No differences appeared between these four bacterial strains in their capacities to nodulate this material. The data also show, despite uneven replication, that $2n(1)$ and to a lesser extent $6n(3)$ displayed a significantly higher degree of resistance even to these strains of bacteria than did $4n(2)$, $6n(1)$, or $6n(7)$. Resistant plants occurred more frequently in the $2n$ than in the $4n$ or $6n$ plant material.

TABLE 3
RESPONSE OF DIPLOID AND HEXAPLOID LINES OF *T. AMBIGUUM* TO INOCULATION WITH AUSTRALIAN AND NEW ZEALAND STRAINS OF *RH. TRIFOLIUM*
ISOLATED FROM NODULES ON *T. AMBIGUUM*

Country in which Strain Isolated	Test Host	Origin of Isolates	Total No. of Isolates	Percentage Isolates Giving:		
				No Nodules in All Four Replicates	Ineffective Response in One or More of the Four Replicates	Effective or Partially Effective Response in One or More Replicates
Australia	2n(1)	Two diploid lines	15	40.0	60.0	0
	2n(1)	Two tetraploid lines	16	12.5	87.5	0
	2n(1)	Four hexaploid lines	29	21.0	75.5	3.5*
	6n(3)	Two diploids (as above)	15	100.0	0	0
	6n(3)	Two tetraploids (as above)	16	62.5	37.5	0
	6n(3)	Four hexaploids (as above)	29	69.0	27.5	3.5*
	<i>T. repens</i> (N.Z. certified)	All as above	60	0	3.4	96.6
	<i>T. subterraneum</i> (Mt. Barker)	All as above	60	0	85.1	14.9
				Percentage of Plants:		
			No. of Replicated Plants	No. of Isolates	Without Nodules	With Effective or Partially Effective Nodules
					With Ineffective Nodules	
New Zealand	2n(1)	One hexaploid line	20	4	100.0	0
	6n(1)	One hexaploid line	44	4	59.1	0
	6n(3)	One hexaploid line	56	4	55.4	0
	<i>T. repens</i> (N.Z. certified)	One hexaploid line	34	4	0	82.4
	<i>T. subterraneum</i> (Mt. Barker)	One hexaploid line	40	4	0	25.0

* The same strain; very slightly effective on 2n host and moderately effective on 6n host.

The effectiveness of the symbiosis was influenced by both host line and bacterial strain. Thus 6*n*(1) responded most effectively with strains A and B, and 6*n*(7), 6*n*(3), and 2*n*(1) less effectively in this order. The bacterial strain D (originally isolated from *T. ochroleucum*) appeared to be the least effective on all lines tested except 6*n*(1), whereas strain B was most effective on this line and was poorly effective on 4*n*(2) and 2*n*(1). Effectiveness is not therefore simply related to the state of ploidy of the host but appears to be determined by complex host-bacterial interactions.

The data entered at the bottom of Table 4 show that the Turkish strains all nodulated both white and subterranean clovers but, unlike the Australasian isolates, were entirely ineffective on both these hosts. *Trifolium fragiferum* L. and *T. ochroleucum* were fully susceptible to these strains and responded ineffectively.

These results showed that certain strains of bacteria from the region of the natural occurrence of *T. ambiguum* were better adapted in symbiosis with this species than strains from elsewhere. Symbiosis with such adapted strains, however, was very variable and achieved a high level of effectiveness in only a small minority of plants.

(ii) *Experiment 2*.—This experiment was designed to provide more detailed information on the symbiosis of two host lines, 2*n*(1) and 6*n*(3), with two Turkish strains of bacteria (A and D). The test plants (22 replicates for each treatment) were grown in agar culture and inoculated when 10 days old. The time at which nodules were formed was noted for each plant and, at harvest, the dry weight of each plant was obtained and its nodules were counted and measured. The results are presented as histograms in Figure 1 in order to emphasize the nature and extent of variations between plants.

Time to nodulation (Fig. 1 (a)) showed a discontinuous distribution for each host and strain, the majority of plants nodulating between 10 and 30 days from inoculation and a small proportion nodulating much later. The late group was larger in the 2*n*(1) line and possibly greater with strain D than with strain A. In all experiments the number of plants which apparently were totally resistant to infection by the Turkish strains was very small. These resistant plants may be considered to comprise the very late-nodulating members of a late-nodulating group. On this interpretation resistance in *T. ambiguum* is not an absolute characteristic but may be regarded as an extreme form of late nodulation.

Weights of the tops of plants grown in tubes for 100 days showed a marked spread (Fig. 1 (b)); in each case there was a distinct low-yielding class comprised of plants ineffectively nodulated or without nodules.

The number of nodules formed upon *T. ambiguum* appeared to be predominantly a function of the host (Fig. 1(c)) and in this experiment was unaffected by bacterial strain. In comparison with the numbers of nodules observed on other species of *Trifolium*, nodules on *T. ambiguum* were everywhere found to be very sparse, particularly upon 2*n* plants. The larger number of infections found with the increase in chromosome complement was in contrast to observations on *Trifolium pratense* L. (Nilsson and Rydin 1954), *T. subterraneum* (Nutman, unpublished data), and *Medicago tribuloides* Desr. (Hely, unpublished data); in these species induced tetraploid plants invariably produced fewer and larger nodules than the diploids. Variation in the number of nodules between plants was observed to be very extensive and to be

TABLE 4

EFFECTIVENESS OF TURKISH STRAINS ON *T. AMBIGUUM* AND FOUR OTHER SPECIES OF *TRIFOLIUM*
GROWN IN TUBES

Host Material	Strain of Nodule Bacteria	No. of Plant Replicates	Percentage of Plants Without Nodules	Percentage of Nodulated Plants Classed as:		
				Ineffective	Intermediate	Fully Effective
2n(1)	A	29	24.1	22.7	54.5	22.8
	B	27	7.4	36.1	39.9	24.0
	C	26	15.4	27.3	45.5	27.2
	D	26	7.7	25.0	29.3	45.7
4n(2)	B	37	0.0	32.5	56.6	10.9
6n(1)	A	16	0.0	6.2	25.0	68.8
	B	16	0.0	0.0	12.5	87.5
	C	16	0.0	6.2	68.8	25.0
	D	16	0.0	12.5	12.5	75.0
6n(3)	A	144	1.4	14.5	65.9	19.6
	B	149	2.0	6.8	60.3	32.9
	C	143	0.0	2.1	81.7	16.2
	D	140	1.4	22.4	64.5	13.1
6n(7)	A	59	0.0	17.0	49.2	33.8
	B	75	0.0	13.3	42.7	44.0
	C	42	0.0	26.2	45.2	28.6
	D	26	0.0	65.4	30.8	3.8
<i>T. repens</i> (N.Z. certified)	A	10	0.0	100.0	0.0	0.0
	B	10	0.0	80.0	20.0*	0.0
	C	10	0.0	90.0	10.0*	0.0
	D	10	0.0	100.0	0.0	0.0
<i>T. subterraneum</i> (Mt. Barker)	A	10	0.0	100.0	0.0	0.0
	B	10	0.0	100.0	0.0	0.0
	C	10	0.0	100.0	0.0	0.0
	D	10	0.0	100.0	0.0	0.0
<i>T. fragiferum</i> (Palestine)	A	20	0.0	100.0	0.0	0.0
	B	20	0.0	100.0	0.0	0.0
<i>T. ochroleucum</i>	B	10	0.0	100.0	0.0	0.0
	D	10	0.0	100.0	0.0	0.0

* Very slightly effective.

proportional to the mean number of nodules formed. In conformation with general experience, nodule size (length) was found to vary inversely with abundance, and was most variable in $2n(1)$ material (Fig. 1(d)).

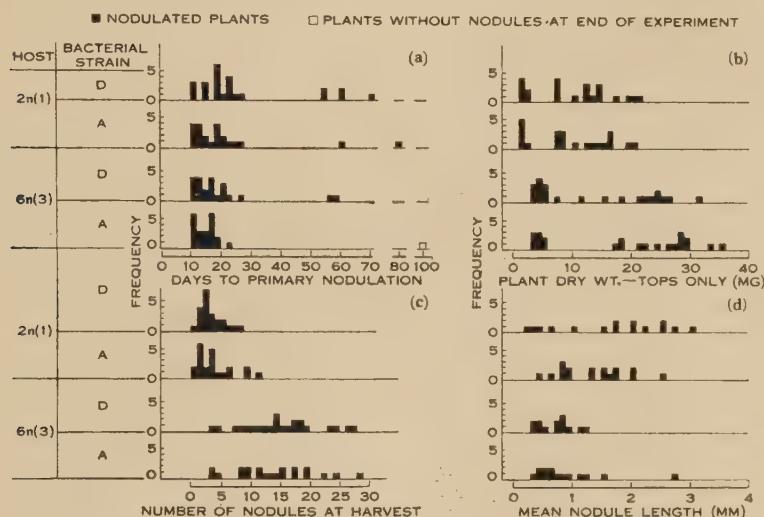


Fig. 1.—Distribution of (a) time to primary nodulation, (b) effectiveness, (c) nodule density, and (d) average nodule size in diploid and hexaploid plants of *T. ambiguum* inoculated with the Turkish strains A or D.

The effectiveness of the symbiosis was also observed to be very variable within each combination of host and bacterial strain and ranged from parasitic associations through all intermediate grades to full effectiveness. No differences were observed between bacterial strains but the $6n(3)$ line responded appreciably more effectively

TABLE 5

DRY WEIGHTS OF $6n(3)$ INOCULATED WITH BACTERIAL STRAIN A AND GROWN IN TUBES FOR 100 DAYS

Effectiveness	No. in Sample	Mean Weight of Tops $\pm$ S.E. (mg)	Mean Total Weight $\pm$ S.E. (mg)
Most effectively nodulated	14	28.93 ± 0.70	42.00 ± 1.30
Partially effective	10	11.60 ± 2.12	22.10 ± 1.99
Ineffective	16	3.44 ± 0.22	9.19 ± 0.40
Resistant	5	4.20 ± 0.20	10.80 ± 1.59
Not inoculated	20	4.00 ± 0.23	11.53 ± 0.86

than the $2n(1)$ and showed also greater range in individual plant size. This is illustrated in Figure 2 which shows the range of response in $6n(3)$ at 60 days. The yield data for similar plants harvested at 100 days from inoculation are given in Table 5. The resistant

plants closely resembled the uninoculated control plants whereas the quite ineffectively nodulated plants were always poorer in top growth than either of the above two classes.



Fig. 2.—Tracing from photograph to illustrate symbiotic variation in *T. ambiguum* 6n(3) inoculated with Turkish strain A, showing the following responses (left to right): (1) most effective observed, (2) partially effective, (3) ineffective, and (4) resistant. Note: (a) kinds of root systems normally associated in the case of tube-grown plants with the abovementioned types of symbiosis, and (b) the distribution, number, and size of nodules characteristic of the differently reacting host plants.

The relations between certain pairs of characteristics of the symbiosis are illustrated in Figures 3 and 4 and disclose a number of interesting correlations. Thus, time to nodulation and nodule number (Fig. 3(a)) were only broadly related, viz. in such a way that abundant nodulation was associated with the early initiation of nodules but not vice versa. Further, the plants nodulating later than 30 days, comprising the distinct group noted above, appeared also to show this relation.

This experiment was terminated arbitrarily at 100 days after inoculation, by which time only two plants were still without nodules. Had it been practicable to extend the experiment it is probable that these two plants also would have nodulated, whereas, had it ended at 50 days, nine plants would have been classified as "resistant". From this point of view resistance can be regarded as a form of extremely sparse nodulation associated with late inception of nodules. These relations will be discussed more fully at a later stage.

The correlation between mean nodule length and nodule density (Fig. 3(b)) is similar to the foregoing. Abundant nodules are invariably small whereas sparse nodules may be large or small. In contrast to the relation between nodulation time and number, no separate groups of observations may be distinguished on the correlation diagram.

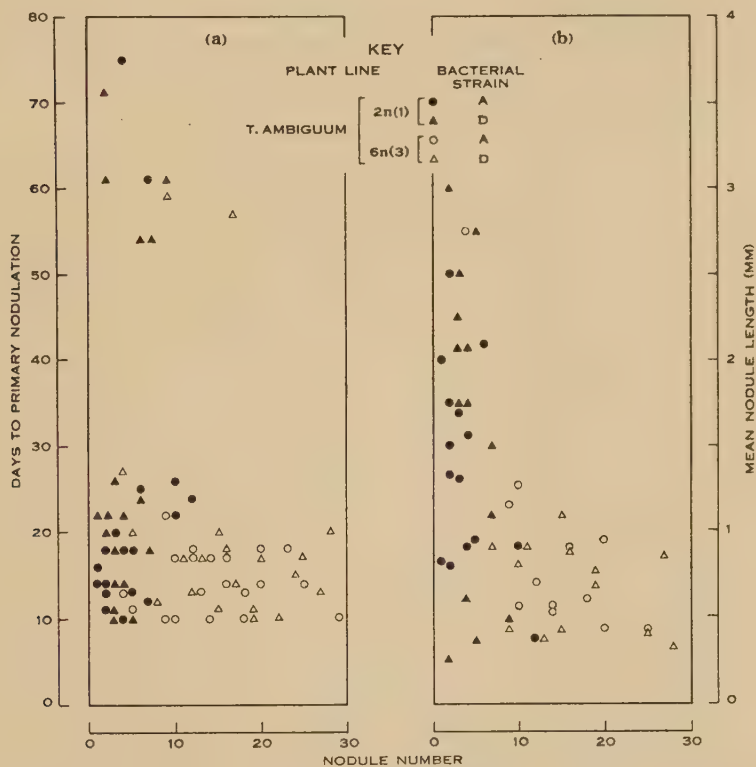


Fig. 3.—Relations between (a) nodule density and time of initiation of nodulation, and (b) nodule density and nodule size.

Effectiveness (as weight of tops) is loosely correlated with nodule number (Fig. 4(a)), the observations again tending to fall into two groups, each including plants from all bacterial-host combinations. These groups were of ineffective plants, on the one hand, and plants showing some degree of effectiveness on the other. This separation is probably related to some broad genetic groupings within the 2n and 6n lines.

Yield was more strongly correlated with average nodule size (Fig. 4(b)) and highly correlated with the total volume of nodules per plant (Fig. 4(c)), as determined from measurements of length and mean diameter of each nodule. At any given yield larger nodules and a larger total volume of nodules were found in the more sparsely nodulating 2n(1) host. The amount of nitrogen-fixing tissue was probably correlated with total nodule volume. Unless the persistence of this tissue varied with size of nodule the above relation suggested that in most plants growth was limited by the

small total volume of nodules produced. Certainly the maximum total volume of *T. ambiguum* nodules (about 12 cu. mm per plant) was much less than found upon *T. subterraneum* growing under similar conditions (about 30 cu. mm).

A comparison of experiments 1 and 2 (although they were carried out at different times) indicated that the properties of the symbiosis are not independent of bacterial strain; in general, the Turkish isolates nodulated earlier and somewhat more frequently and effectively than the others. To confirm these differences under the same conditions a further experiment was undertaken to investigate, in particular, the relation between delayed susceptibility and resistance.

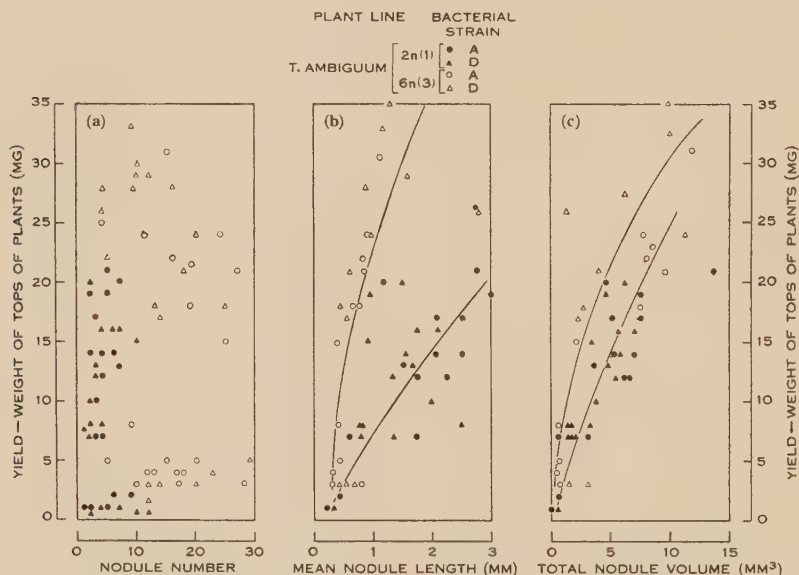


Fig. 4.—Relations between yield and (a) nodule number, (b) nodule length, and (c) total nodule volume.

(iii) *Experiment 3*.—Three lines of *T. ambiguum* were employed in this test—2n(1), 6n(1), and 6n(3). The plants were grown in agar culture and were inoculated with either the Turkish or New Zealand isolates of bacteria. Sets of *T. repens* and *T. subterraneum* were also included for comparative purposes. Daily records were taken of the presence or absence of nodules from the 8th until the 25th day, and at about 80 days the plants were taken from the tubes and finally scored for resistance or susceptibility. The results are given in Figure 5. In confirmation of the earlier experiments these results revealed marked differences ascribable to strains and also clearly demonstrated the connection between late nodulation and “resistance” in *T. ambiguum*. Thus the symbiosis of 6n(1) × Turkish strains nodulated earlier than any others, contained only one plant nodulating later than 25 days, and showed no resistant plants. With the 6n(3) × Turkish strains combination, nodules arose on the majority of plants over a somewhat longer period, a small group of four plants

nodulated later than 25 days, and one plant was still without nodules at the end of the experiment. The most tardy nodulation by the Turkish strains was with the 2n(1) plant material. With this, nodule production began on the 14th day and then continued at a fairly uniform rate until the 22nd day. Thereafter nodules continued to be produced at irregular intervals until the end of the experiment, at which time 12 plants were still without nodules.

With the New Zealand isolates, nodulation occurred much later throughout and the proportion of resistant plants was much increased. As with the Turkish strains, 6n(1) nodulated on the average earlier than 6n(3), and gave rise to fewer plants which remained unnodulated until the end of the experiment. Also 2n(1), which produced the largest proportion of resistant plants with the Turkish strains,

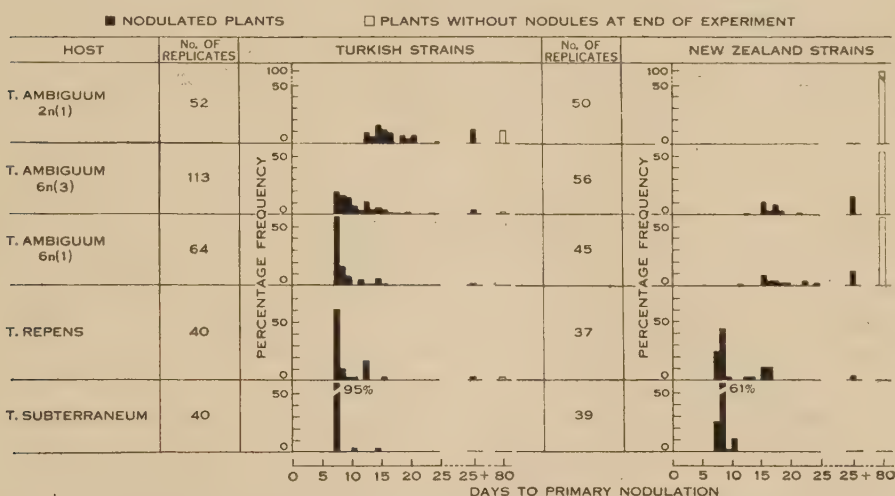


Fig. 5.—Distribution of time to primary nodulation in *T. ambiguum*, *T. repens*, and *T. subterraneum* with Turkish and New Zealand strains of nodule bacteria.

was wholly resistant to the New Zealand isolates under these experimental conditions. The distribution of time to nodulation tended in all cases to be discontinuous, as was observed in experiment 2, and to consist of a main part and a secondary distribution extending over a long period and separated from the first by an appreciable interval of time. These results support the conclusion drawn from the earlier experiment that the resistant plants belong to that part of the host population which becomes susceptible to infection only at a late stage in growth. In further confirmation it was observed that 15 surviving "resistants" of 6n(3) planted out into soil all eventually produced a few nodules.

A comparison of nodulation time in *T. ambiguum*, *T. repens*, and *T. subterraneum* (Fig. 5) suggests that this kind of resistance occurs to varying degrees in the three species. Thus *T. repens* nodulated over a fairly extended period and gave rise to a single resistant plant. The occurrence of occasional resistant plants in *T. repens* has also been noted in other experiments. *T. subterraneum* nodulates much more

promptly and only very rarely fails to form nodules under these conditions. Selection experiments carried out with *T. pratense* (also a cross-pollinated species) have shown that earliness and lateness of nodulation are inherited in the host in a complex manner (Nutman 1953).

IV. DISCUSSION

These results emphasize the very heterogeneous behaviour of *T. ambiguum* in symbiosis. This may be due wholly or in part to heterozygosity associated with an obligatory cross-fertilizing habit. It is qualitatively similar to, although more extensive than, the symbiotic variability described for red clover, which is also a cross-fertilized species (Nutman 1946). The unusually high degree of variation encountered in the particular introductions which have been investigated may have been augmented by inbreeding at some stage during the multiplication of stocks from small original seed samples. Alternatively, heterogeneity in *T. ambiguum* may reflect a more fundamental genetic instability associated with the differentiation of the $2n$, $4n$, and $6n$ races.

(a) Resistance

Genetic heterogeneity has been suggested as the cause of failure of individual plants to form nodules during the period of an experiment, particularly with some strains of bacteria. In this context, "resistant" plants are those which exhibit a very delayed susceptibility to infection possibly combined with very sparse infectibility, probably also genetically determined. Breeding experiments are in progress to examine the interrelations between lateness, sparseness, and resistance. This hypothesis is compatible with the variation observed in the host's resistance to infection by different bacterial strains and with the established effect of grafting.

The interpretation of quantitative resistance in physiological terms raises a number of interesting questions, some of which may be verified experimentally. In *T. pratense* it has been shown that infection does not occur at random upon the root but is restricted to discrete foci, each of which is available for infection over a limited period of time (Purchase and Nutman, unpublished data). It is possible that in *T. ambiguum* such foci are not only fewer in number but also are open to infection for shorter periods than in other species. Bacterial strain differences might then be due to the rapidity with which the preliminary phases are completed.

This kind of resistance is in contrast to the examples of complete resistance to infection described for red clover (Nutman 1949) and soybean (Williams and Lynch 1954). These have been shown to be associated with gene mutation at single loci and in the case of red clover (Nutman 1949) and soybean (Hely, unpublished data) to be incapable of modification by grafting.

Resistance in *T. ambiguum* may be more allied to the relative resistance found in plants of one cross-inoculation group to infection by bacteria from another. In fact the data provided by the $2n$ plant material and New Zealand and Turkish isolates would be sufficient to constitute a *T. ambiguum* cross-inoculation subgroup, similar to the subgroupings proposed in the cowpea group (Johnson and Allen 1952).

(b) *Effectiveness*

The patterns of symbiotic response found among species of *Trifolium* in symbiosis with strains of *Rh. trifolii* have been investigated extensively. From this, Vincent (1954) concluded that the species of *Trifolium* comprise three groups showing broadly similar responses. The first includes *T. repens* and *T. pratense*, the second *T. subterraneum* and *Trifolium incarnatum* L., and the third only *T. ambiguum*. The results here presented support this classification since isolates from the type species of both these groups were ineffective upon *T. ambiguum* whereas the Turkish strains, partially effective upon *T. ambiguum*, were wholly ineffective upon the type species of the first two groups.

Ineffectiveness in *T. ambiguum* and resistance appear to be correlated, in that bacterial strains which are least able to form nodules are most ineffective. This is probably a fortuitous relationship and does not necessarily indicate a genetic connection between the host factors responsible for resistance and ineffectiveness. Although the mechanism of adaptation between bacteria and plant is very little understood it would seem probable, from what is known of the general biology of the symbiosis, that a time sequence of interactions between the symbionts is involved, the early ones concerning infection and nodule formation and the later ones bacteroid formation and nitrogen fixation. It is therefore reasonable to assume that associations of bacteria and host ill-adapted for the preliminary stages would be necessarily poorly adapted for the later ones. In *T. ambiguum* adaptation is clearly inadequate at a number of stages: at infection, in the induction of a sufficient amount of nitrogen-fixing tissue, and probably also in the efficient functioning of this tissue. In this connection it may be recalled that in the rare instances of nodule formation on a member of a well-defined cross-inoculation group by bacteria from another group, the nodules are invariably ineffective (Kleczkowska, Nutman, and Bond 1944).

(c) *Polyploidy*

The effect of chromosome number on the symbiosis was not well defined. The $2n$ host appeared to be generally less susceptible and less effective than $4n$ or $6n$ plants, between which much unsystematic variation occurred. The correlation between polyploidy and a reduction in infection rate, observed in other species, was not found. On the contrary the hexaploid races were the most freely nodulating. If, however, resistance is due, as suggested above, to the accumulation of recessive factors for sparseness and lateness of nodulation, an increase in the chromosome number would lead to a reduction in the proportion of recessive homozygotes.

V. ACKNOWLEDGMENTS

Sincere thanks are extended to Dr. E. A. Hollowell and Dr. L. W. Erdman, United States Department of Agriculture, Beltsville, U.S.A., and to Mr. R. M. Greenwood, D.S.I.R., Palmerston North, N.Z., for provision of seed and cultures; also to Mr. W. Hartley, Dr. R. D. Brock, and Mr. J. Brockwell, all Division of Plant Industry, C.S.I.R.O., Canberra, who respectively supplied seeds, determined chromosome numbers, and assisted materially with tube experiments. Dr. P. S. Nutman, Senior Research Fellow with this Division, encouraged and guided the investigation and provided valuable help in the preparation of the manuscript.

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THE OCCURRENCE OF A PREVIOUSLY UNOBSERVED POLYSACCHARIDE IN IMMATURE INFECTED CELLS OF ROOT NODULES OF *TRIFOLIUM AMBIGUUM* M. BIEB. AND OTHER MEMBERS OF THE TRIFOLIEAE

By F. J. BERGERSEN*

[Manuscript received July 9, 1956]

Summary

A type of ineffective nodulation is described in which the bacteroids in the nodule cells fail to mature, and in which the infected host cells accumulate water-soluble polysaccharide between protoplast and cell wall. Ineffectiveness of this kind is characteristic of nodules on *Trifolium ambiguum* M. Bieb. produced by unadapted strains of bacteria, or produced by adapted strains on a small proportion of plants. It is also found when strains effective with *T. ambiguum* nodulate subterranean or white clover. The polysaccharide in the peripheries of infected cells is readily seen by phase-contrast observation, provided the sections are not hydrated after removal of wax, but it is not visible by ordinary staining procedures.

Examination of a number of effective nodules from various members of the Trifolieae revealed that the above type of polysaccharide accumulation always occurs as a transitory deposit in a narrow layer of cells across the immature region of the bacteroid-containing tissue. The possible significance of this deposit is discussed.

I. INTRODUCTION

A study of the symbiosis of *Trifolium ambiguum* M. Bieb. in progress in this Laboratory has revealed very extensive variation in the host, which particularly affects nodulating capacity (i.e. susceptibility to infection), and the symbiotic effectiveness of the nodules produced by adapted and unadapted strains of nodule bacteria. The extreme expression of variation found in this species is thought to be related to its cross-fertilizing habit (Hely 1956).

With adapted strains of bacteria (i.e. those obtained from the region of natural occurrence of *T. ambiguum*) a largely effective symbiosis was established, only a small proportion of the plants remaining nitrogen deficient. In some of the latter the nodules were found to be too few and too small to be able to effect any detectable nitrogen fixation, but in others the number of nodules produced was larger and an appreciable amount of potentially fixing tissue was formed. Most Australian isolates of *Rhizobium trifolii* either failed to nodulate *T. ambiguum* or did so in a very sparse and ineffective manner.

Ineffective nodules of these kinds have been examined by various cytological and cytochemical procedures, and their structure compared with that of effective nodules on *T. ambiguum* and nodules produced by the same strains of bacteria on other *Trifolium* species, as well as with nodules produced by known effective strains of bacteria on *Trifolium* and *Medicago* species.

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II. MATERIALS AND METHODS

(a) *Plant and Bacterial Material*

Nodules were examined from plants grown in test-tube culture after the method of Thornton (1930), and from inoculated plants grown in pots. For the most part the hexaploid, $6n(3)$, strain of *T. ambiguum* (Hely 1956), and commercial samples of *T. subterraneum* L. (var. Mt. Barker), *T. repens* L. (New Zealand certified mother seed), and *Medicago tribuloides* Desr. were used. Nodules from *M. hispida* J. Gaertn. grown in plots at Temora, N.S.W., were also compared with the others. The various

TABLE 1
PLANT AND BACTERIAL MATERIALS USED AND TYPE OF SYMBIOSIS ESTABLISHED

Host	Bacterial Strains	Type of Nodules Examined
<i>T. ambiguum</i> ($6n(3)$)*	A*	Effective and ineffective
	B*	Effective and ineffective
	C*	Effective and ineffective
	D*	Effective and ineffective
	Australian isolates	Ineffective
<i>T. subterraneum</i> (var. Mt. Barker)	A	Ineffective
	B	Ineffective
	C	Ineffective
	D	Ineffective
	SU297	Effective
<i>T. repens</i> (New Zealand certified mother seed)	A	Ineffective
	B	Ineffective
	C	Ineffective
	D	Ineffective
	C1F†	Effective
<i>M. tribuloides</i>	CC146	Effective
	CC157	Effective
<i>M. hispida</i>	Nodules from the field, Temora, N.S.W.	Effective

* See Hely (1956).

† Rothamsted culture collection.

host-bacterial strain combinations examined are set out in Table 1. Cultures were maintained on yeast-water-mannitol-agar, and plants were inoculated immediately after germination. Potted plants were grown from plants initially cultured in test tubes where their symbiotic response had been determined.

(b) *Microscopic Methods*

Nodules were fixed in Flemming's solution, dehydrated in graded ethanol, and embedded in paraffin wax. For unstained preparations the following procedure was employed. Sections $3-5\mu$ in thickness were cut and mounted on slides, the wax was removed by two changes of xylol, and the sections mounted under thin

coverslips in "Xam" (Gurr). These were examined with a "Wild Varicolor" phase-contrast system using monochromatic green light, the exact wavelength (between 500 and 530 m μ) being selected for each preparation to give minimal refractility of cell wall material and maximal contrast in the various cytological features being examined. Such changes in wavelength as were necessary were effected by tilting the interference filter in the light source of the equipment. The principles of this method are discussed by Dufour and Locquin (1951).

Normal bright-field illumination was used for stained preparations and sections were cut with a thickness of 5–7 μ . Rose bengal–light green (Brenchely and Thornton 1925) was used as a general anatomical stain and polysaccharide was stained by the Hotchkiss method (Glick 1949).

Bacteroids from nodules were examined as previously described (Bergersen 1955) using phase-contrast observation and staining with tetrazolium blue or neotetrazolium chloride (Synthetical Laboratories, Chicago, U.S.A.) in M/15 phosphate buffer

Photomicrographs were obtained with a "Mikas" camera attachment using Ilford "Micro-neg Pan" film and processing for maximum gradation.

III. RESULTS

(a) *Nodule Structure in T. ambiguum*

Stained sections showed that there were no gross structural abnormalities in the ineffective nodules of *T. ambiguum*. The decay of the bacteroid-containing tissue took place somewhat earlier in ineffective than in effective nodules, but the bacteroid-containing tissue persisted for some time in the former. The degree of persistence of the bacteroid-containing tissue was independent of the kind of ineffectiveness produced, whether associated with adapted or unadapted strains of bacteria.

Phase-contrast methods, however, revealed marked histological differences between effective and ineffective nodules, particularly with regard to carbohydrate accumulation. In all ineffective nodules excessive accumulation of starch occurred: this appeared as very bright grains. It was also observed that the boundary of the protoplast of the nodule cells containing ineffective bacteroids was brightly refractile compared with the same structures in effective nodules. Closer examination showed that this refractility was due to deposition of material between protoplast and cell wall of the infected cells (Plate 1, Figs. 2, 3, and 4). The amount of deposit paralleled starch accumulation which occurred in adjacent, uninfected cells; the material appeared first as refractile granules at the periphery of the young infected cells into which the bacteria had been recently released, had multiplied, and were undergoing the first stages of bacteroid formation. These deposits of refractile material coalesced as development of the host cell proceeded, and eventually formed a more or less complete layer around the bacteroid-filled cells. Deposition was much greater at peripheries adjacent to uninfected cells than between infected cells (Plate 1, Figs. 2, 3, and 5).

Because of the similar refractile nature of the starch grains and the deposited material it was thought that this material was probably also polysaccharide in

nature. Preparations, stained by the Hotchkiss method, were made using 70 per cent. (v/v) alcoholic solutions to prevent possible loss of the deposited material during the staining procedure due to solubility in water. These tests confirmed the polysaccharide nature of the material (Plate 1, Fig. 5). Comparison of sections stained by the rose bengal-light green method and unstained sections, seen by the phase-contrast method, showed that some of the deposited carbohydrate was removed during staining in aqueous solutions, and that which remained was unstained. It is doubtless for this reason that this material has not previously been reported. Experiments were then performed which showed that the polysaccharide was almost entirely removed from sections by immersion for 1 hr in water at 30°C: a treatment which did not alter the appearance of the starch in these sections. In sections mounted in Lugol's or Gram's iodine the starch was stained blue and the peripheral polysaccharide brownish blue. From these observations it was concluded that the polysaccharide deposited in the periphery of infected cells of ineffective nodules from *T. ambiguum* was in a fairly highly polymerized form, possibly a dextrin.

(b) *Structure of Nodules Produced by Strains A, B, C, and D on Subterranean and White Clover*

Observations were next extended to an examination of ineffective nodulation produced by the *T. ambiguum* bacteria strains A, B, C, and D on subterranean and white clovers. Sections of such nodules revealed the same persistence of bacteroid-containing tissue as occurred with the ineffective *T. ambiguum* nodules. This tissue was similar in appearance in the three clovers, and also accumulated carbohydrate at the periphery of infected cells. This phenomenon would therefore appear to be general in ineffective *Trifolium* nodules produced by these strains of *Rhizobium*.

(c) *The Transitory Accumulation of Carbohydrate in Effective Nodules in the Trifolieae*

Closer examination of effective nodules on *T. ambiguum* by the phase-contrast method showed that a limited amount of peripheral carbohydrate also occurred in actively nitrogen-fixing nodules. It was, however, limited to a narrow zone of cells a short distance behind the nodule meristem and within which bacteroid formation was not yet complete. In section, the cells containing the peripheral carbohydrate appeared as a narrow band transverse to the main axis of the nodule and were one or two cells in thickness (Fig. 1).

An examination of effective nodules on subterranean and white clovers was then undertaken together with *Medicago tribuloides* and *M. hispida* nodules. In all these species a band of cells was again found which contained immature bacteroids and a deposit of peripheral polysaccharide (Plate 1, Fig. 6).

(d) *Peripheral Carbohydrate Accumulation and Immaturity of Bacteroids*

The association of a layer of carbohydrate with a certain stage of bacteroid development suggested that the uniform distribution of peripheral carbohydrate throughout the ineffective nodules on *T. ambiguum* may be associated with a persistence of a juvenile stage of bacteroid formation.

Examination of *T. ambiguum* showed that the bacteroids from ineffective nodules were, in fact, identical with bacteroids from very young effective nodules: morphologically in that they were much thinner and metabolically in possessing fewer oxidatively active granules than mature effective bacteroids (Fig. 2), as shown by staining with tetrazolium compounds in the presence of a substrate such as glucose or succinate. Phase-contrast observation suggested that the perinuclear areas found in effective subterranean clover bacteroids (Bergersen 1955) were also absent from the immature bacteroids. These findings suggested that the bacteroid-containing tissue found in ineffective nodules of *T. ambiguum* is unable to fix nitrogen because the bacteroid development is incomplete.

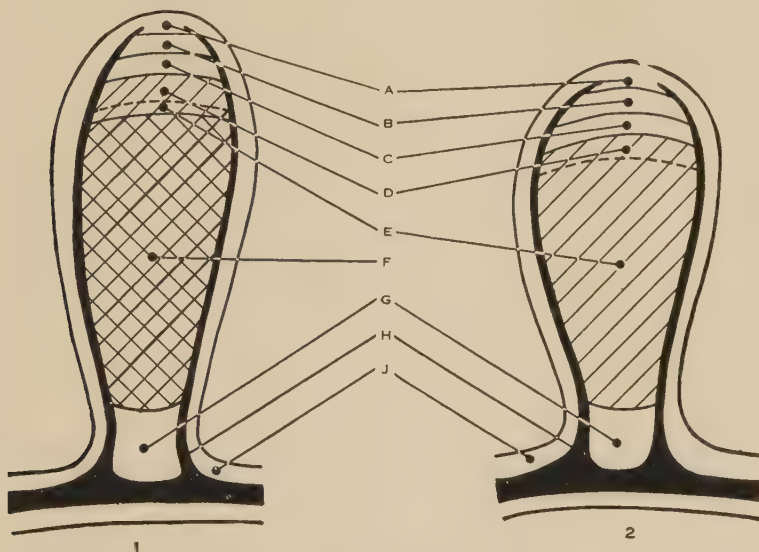


Fig. 1.—Diagrammatic representation of the arrangement of tissues in ineffective (1) and ineffective (2) nodules in *T. ambiguum*. A, meristem; B, region of infection thread penetration; C, release of bacteria from threads and their intracellular multiplication; D, region of bacteroid formation; E, region of cells containing immature bacteroids and peripheral polysaccharide; F, region of active, mature, bacteroid-containing tissue; G, decay of old tissue; H, vascular system of nodule; J, cortex of nodule and root.

IV. DISCUSSION

The existence of a zone of cells in which polysaccharide accumulates between cell wall and protoplast is a hitherto undescribed feature in the development of effective nodule tissue, and, together with the persistence of these cells in ineffective nodules of the *T. ambiguum* type, invites consideration of its relation to the nitrogen-fixing processes. In ineffective nodules absence of nitrogen fixation appears in all cases to be correlated with the failure of the bacteroids to complete their development. This failure may be caused by inadequacy of the host plant or of the bacteria since some strains of bacteria gave both effective and ineffective nodules on *T. ambiguum* and entirely ineffective nodules on two other host species. From

this and previous work (Bergersen 1955, Bergersen and Nutman 1957) it has become clear that successful symbiotic nitrogen fixation depends on the completion of a series of steps, each of which involves interactions between host cells and bacteria. The failure or blockage of any of these steps prevents fixation. This concept has already been stated in a preliminary report (Bergersen 1956). In the present case evidence points to the failure of a fairly late step in which some essential change in the bacteria has not taken place.

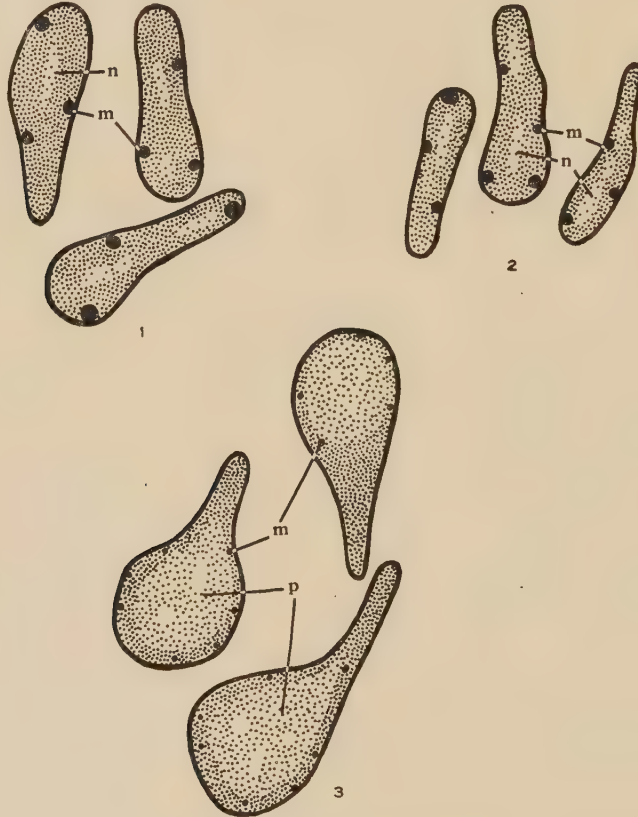


Fig. 2.—Drawings of bacteroids seen by phase-contrast microscopy. 1, Bacteroids from a very young effective *T. ambiguum* nodule. 2, Bacteroids from an ineffective *T. ambiguum* nodule. 3, Bacteroids from mature tissue of an effective *T. ambiguum* nodule. *n*, Site of nuclear material; *m*, metabolically active mitochondria-like granules; *p*, perinuclear region of mature bacteroids.

The occurrence of carbohydrate as a transitory deposit in the periphery of infected cells of effective nodules on a number of host plants suggests the following hypothesis.

Host tissues provide appropriate intermediate substances which are used as substrates for the metabolism of the bacteroids inside infected cells. Some of these substances are transformed into amino acids etc. by combination with the primary compound of nitrogen fixation. The intermediate substances appear to be

provided before the bacteroids are fully matured and are synthesized into a temporary reserve material until fixation commences within the cells. The arrangement of the polysaccharide supports this hypothesis, since it is most abundant adjacent to uninfected cells and least abundant, or even absent, between infected cells: i.e. it occurs in regions in which one would expect to find accumulation if its utilization cannot take place.

The bacteroid-containing tissue consists of cells which have twice the chromosome number of uninfected tissue (Wipf and Cooper 1940). Differences in osmotic relations and permeability to non-electrolytes have been found between diploid and tetraploid plant tissues (Kostov and Nikolov 1941; Chen and Tang 1945; Chandler and Barton 1955): such differences may be a necessary condition for the passage of chemical substances into bacteroid-containing cells, and the carbohydrate accumulation may be a function of the relative ploidy of adjacent infected and uninfected cells. Further work on the nature of substances supplied by the host plant to bacteroid-containing cells may prove of value in elucidating more of the details of symbiotic nitrogen fixation in root nodules on leguminous plants.

The methods described have been used for many years with the exception of the phase-contrast technique; this has several noteworthy advantages:

- (i) It renders visible nodule structures which required at least two different staining methods to demonstrate.
- (ii) By eliminating the aqueous staining stages, loss of water-soluble material is minimized, although care should be taken in the interpretation of the appearance of precipitated water soluble substances.
- (iii) It proves very useful as a screening method for the examination of large numbers of preparations, since it eliminates hydration, staining, and dehydration of the sections. The general aspect of tissues at first seems unfamiliar, but with practice, all histological features can be readily recognized.

V. ACKNOWLEDGMENTS

The author wishes to thank Mr. F. W. Hely, Division of Plant Industry, C.S.I.R.O., for supplying plant material, and Dr. L. W. Erdman, United States Department of Agriculture, for cultures. Dr. P. S. Nutman has given valuable criticism and encouragement.

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EXPLANATION OF PLATE I

Figures 1, 2, 3, 4, and 6 are 'Varicolor' photomicrographs of unstained nodule sections, 3–5 μ in thickness, and photographed in light of wavelength 500–530 m μ . All magnifications $\times 1400$.
b, Bacteroid-filled host cytoplasm; p, peripheral polysaccharide; s, starch

Fig. 1.—Effective *T. ambiguum* nodule—bacterial strain A: bacteroid-filled cells.

Fig. 2.—Ineffective *T. ambiguum* nodule—bacterial strain A. Showing deposition of refractile polysaccharide in bacteroid-filled cells.

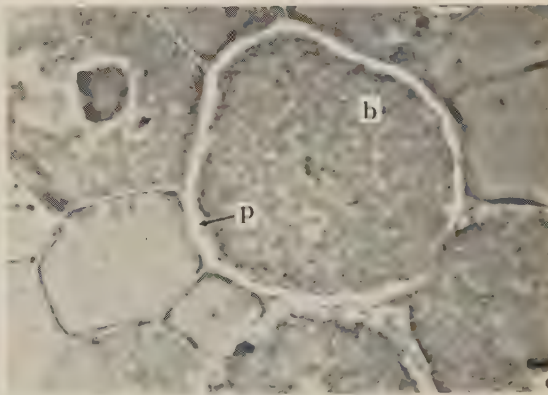
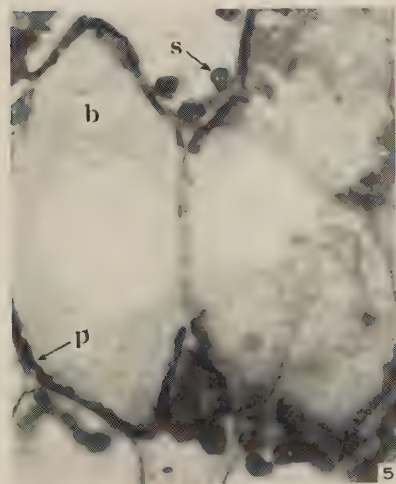
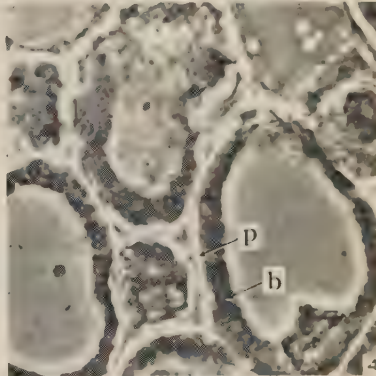
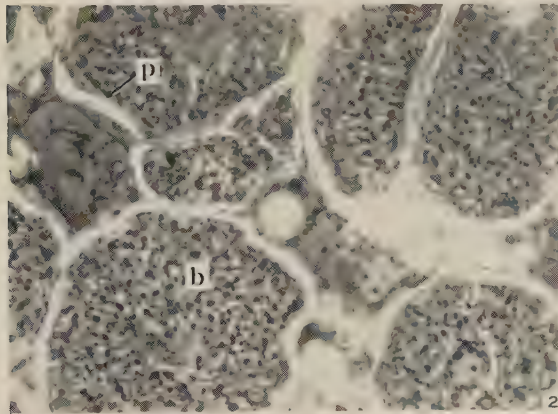
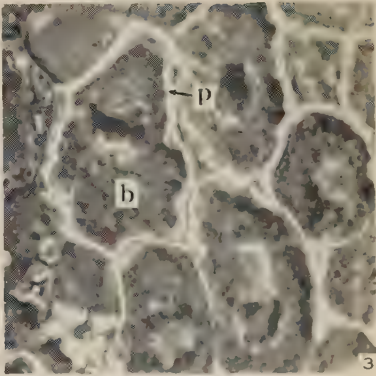
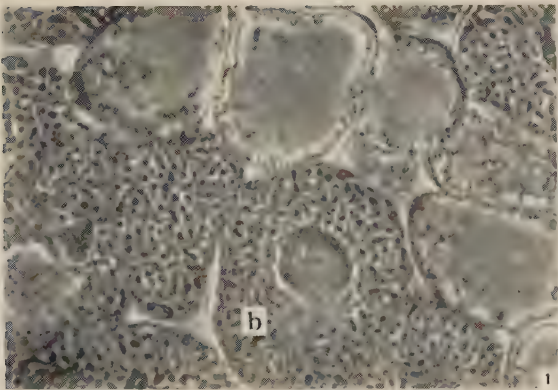
Fig. 3.—Ineffective *T. repens* nodule—bacterial strain A: as for Plate 1, Figure 2.

Fig. 4.—Ineffective *T. subterraneum* nodule—bacterial strain A: as for Plate 1, Figure 2.

Fig. 5.—Ineffective *T. ambiguum* nodule: Hotchkiss strain of bacteroid-containing tissue. Starch and peripheral polysaccharide stained dark red.

Fig. 6.—Effective *M. tribuloides* nodule—bacterial strain CC146: polysaccharide deposition in the periphery of a layer of cells, one cell deep, across the nodule tip. The meristem is out of the picture to the left and mature bacteroid-containing tissue is to the right.

POLYSACCHARIDE IN ROOT NODULES



TRASHY LEAF IN AUSTRALIAN-GROWN FLUE-CURED TOBACCO: THE LIGHT FACTOR IN THE DEVELOPMENT OF AMENENIC LEAF

By R. JOHANSON*

[*Manuscript received July 9, 1956*]

Summary

Artificial shading of mature plants of a flue-cured variety tobacco in the field 4–14 days before harvesting produced 22 per cent. loss in weight of leaves. Their labile hexose-carbohydrate content was about 64 per cent. less than that of control leaves; when cured, shaded leaves were trashy. In plants receiving extra sunlight the weight per unit area of leaves was increased by 20 per cent., and their carbohydrate content by 45 per cent; there were no trashy leaves with this treatment. Control leaves occupied an intermediate position between shaded and extra-light leaf in composition and quality.

On an absolute basis, treatments virtually had no effect on total and protein nitrogen, but, on a dry weight basis, shaded leaf had about twice as much nitrogen as that of leaves with extra light. Shading of leaves reduced the average ratio of carbohydrate to nitrogen (curing index) to one-third as compared with the controls, and produced leaf with poor flue-curing properties; such leaf loses yellow pigments and other desirable compounds during the starvation period of curing.

Green leaves have considerable capacity to absorb light; as little as 3 per cent. may pass through a single leaf. Tobacco leaves being entire may contribute extensively to poor light conditions and internal shading. It is concluded that light is often a limiting factor in dense crops and during periods of cloudiness, thus contributing to production of trashy leaf.

I. INTRODUCTION

With cigarette tobacco it is essential that harvested leaf should have the correct composition if it is satisfactorily to undergo flue-curing. Deficiency of labile hexose carbohydrates relative to nitrogen fractions characterizes amenenic leaf (Johanson 1954b); this leaf is succulent, it has less dry matter per unit area than the corresponding non-amenenic leaf, and it is unsuitable for curing. When cured, amenenic leaf loses yellow pigments, has low capacity for moisture, and becomes brown, brittle, and trashy (Johanson 1951). Both trashy leaf and quickly dried amenenic leaf fluoresce only faintly or not at all under ultraviolet light. Any fluorescence tends to be confined to the veins, whereas good leaf fluoresces brilliantly on the underside (Johanson 1953).

In the field it has been noticed that amenenic leaf comes mainly from well-developed plants where the leaves are extensively shaded, and from plants which have a high nitrogen status. In some crops where shading of leaves is prevalent, exceedingly low light intensities have been measured. These may be of the order of 150 f.c. in the shade between the rows, and between the plants as little as 20 f.c. on days when full sunlight was from 8000 to 10,000 f.c.

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In this paper, study was made of the influence of light on quality of tobacco leaves and the data are discussed in relation to the naturally occurring field shading and cloudiness. Results are presented of experiments with glass-house plants grown at Canberra, and with field plants in north Queensland, where they were subjected to artificial shading and extra sunlight to determine the effect on their leaf composition and curing properties.

II. EXPERIMENTAL

(a) *The Shading Experiment*

A block of tobacco plants of the flue-cured variety Hicks was selected for uniformity from a commercial crop at Ayr, north Qld. The eight rows chosen were in a north-south direction, were 124 ft long and 4 ft apart. The plants were 21 in. apart within the rows. The crop was of average growth for the district and plants were topped at 5 ft 6 in. high.

(i) *Shaded Plants (S).*—The aim was to accentuate field conditions under which shading of the lower portions of the plants by leaves from adjacent plants and rows occurs when vigorously grown crops approach maturity. The shading was set up 4 days before the first harvest by spreading hessian covers horizontally on wires between two rows at a height of 4 ft 6 in. and by stretching narrower sloping covers on the outer sides of these two rows. The tops of the plants protruded through the 6-in. gaps between the parallel strips of hessian. The average light intensity between the unshaded rows was 8400–9500 f.c. at noon, and between hessian-shaded rows this was reduced by about 88 per cent. The ground and air temperatures under the plants showed no difference between hessian-shaded and non-shaded rows.

(ii) *Plants with Extra Light (L).*—Four days before the commencement of the first harvest every second plant of the two L rows was carefully bent at the roots making a 30°-angle with the surface of the ground. With this increase in spacing to 42 in., shading of lower leaves by adjacent plants was effectively reduced and full sunlight was available to the standing plants most of the day, competition for water and nutrients, however, remaining virtually the same as in the control rows.

(iii) *Control Plants (C).*—In the untreated control rows, the lower leaves experienced some shade, particularly in the morning and evening periods. Although there were four control rows in the original design only two were used for analysis, because no significant differences could be detected in the composition of leaves from random samples taken from these rows.

(iv) *Harvesting, Curing, and Drying of Leaf.*—A few days before harvesting, the leaves near to the ground were examined for ripeness and general condition and the four lowest leaves which could have been used in commercial harvesting up to this time were discarded. Experimental harvesting commenced with the fifth tier of leaves on December 8, 1950, and continued for 10 days; each harvest was started at about 10 a.m. The rate of maturation (yellowing of petioles and general yellow cast) of the crop was rapid and several tiers of leaves were picked on each harvesting day. To keep them separate, however, one tier was harvested at a time. From each tier a leaf from every second plant was taken for curing, the remainder being quickly dried in barns at 75–80°C. The leaves for curing were traced for area then strung on sticks and placed with commercial crops in barns for curing.

(v) *Sampling and Grading*.—The cured leaves from each tier and each treatment were graded into good (non-papery, uniformly yellow), semi-trashy (papery with brown patches), trashy (papery and completely brown), and green (showing any green cast).

The bulk samples of each tier of quickly dried uncured leaf were subsampled; blades of alternate leaves were stripped from the midrib, crushed, mixed, coned, and quartered. One-quarter was ground and prepared for analysis.

(b) *Glass-house Experiments*

(i) *Leaves for Light Absorption and Transmittance Measurements*.—Groups of 20 tobacco plants (var. Virginia Gold) were grown in a glass-house at Canberra, in two positions. The average light intensities in these positions were 2000 and 6000 f.c. at noon in September and October 1954. All leaves from mature plants of each group were sorted into green, light green, and yellow-green categories; these bulk samples were subsampled, leaves typical of each colour group being selected.

Simple measurements of light intensity were made with a Weston light-meter and a Weston Master meter, both calibrated against a known reflectance surface. Measurements of transmittance and of reflectance were made with a G.E. recording spectrophotometer.

(ii) *Leaves for Studies of Curing and of the Effect of Excessive Soil Nitrogen*.—Tobacco plants of the flue-cured variety (Virginia Gold) were grown in a glass-house at Canberra, in 10 in. pots, one plant per pot, using a soil known to produce healthy plants free from symptoms of nitrogen deficiency. Flower buds and suckers were removed by hand.

At the time when plants had differentiated 22 leaves, 24 plants were selected for uniformity and were divided at random into three groups of eight. One group served as control (N_0), and received no extra nitrogen. The second group (N_1) received 4.3 g and the third group (N_2) 8.6 g of KNO_3 per plant in a solution during 1 week's watering. As the leaves matured, they were removed and divided at random into two groups; one group was quickly dried and the other was cured.

(iii) *Curing Method*.—Leaves, pinned through the midrib onto soft sticks, were placed at $38 \pm 1^\circ C$ in an oven where relative humidity during the initial starvation period was maintained at 98–100 per cent. After about 3 days, when most of the chlorophyll had disappeared, humidity was reduced to that of the room. Under these conditions the portion already yellow dried out, while some chlorophyll remaining in the midrib and in the other areas still green continued to break down. As soon as the leaf web was uniformly yellow, or had lost all its green colour, the temperature was raised to $42 \pm 1^\circ C$ for about 10 hr. After loss of all green colour from the midrib, the temperature was increased to about $65^\circ C$ for 2–3 hr and finally to $80^\circ C$ for 4 hr.

(c) *Chemical Methods*

(i) *Carbohydrates*.—Hydrolysis of powdered material with 0.7N H_2SO_4 (Shmuk 1948) yields a fraction which in this paper is called "labile hexose carbohydrate"; this includes soluble hexoses, starches, dextrans, and some non-fermentable carbohydrate.

Dry leaf powder (0.2–0.5 g) is hydrolysed with 50 ml 0.7N H_2SO_4 in a 250-ml conical flask in a boiling water-bath for 45 min; hexoses, as determined by anthrone, do not increase on further heating. The flask contents are cooled to 0°C , filtered, and further cooled while neutralizing with N NaOH (about 35 ml). To clarify, 15 ml of M ZnSO_4 is now added followed by N NaOH (23–25 ml) with constant stirring until just alkaline to phenolphthalein, and the mixture filtered under slight vacuum through Whatman No. 1 paper covered with a 1-cm layer of water-washed cotton wool. The filtrate is finally treated with N H_2SO_4 (about 0.5 ml) until opalescence disappears, and hexoses are determined by the anthronation procedure of Johanson (1954a).

In one instance, soluble hexoses were removed during the course of a protein preparation (see below) and “reserve hexose carbohydrate” was determined after hydrolysis of the residue by 0.7N H_2SO_4 .

(ii) “*Protein*” *Preparations*.—These were made and nitrogen determinations carried out as described by Johanson (1951); “extractables” represent the material extracted by the 80 per cent. ethanol used in this method.

III. RESULTS

(a) *Effect of Shading*

After 2 days shading, the undersides of the shaded leaves became progressively smoother and less gummy, and gradually acquired a pale green colour and glossy texture; the leaves were soft and thin to the touch, and somewhat succulent. On the other hand, leaves of plants fully exposed to the sunshine were gummy, thicker to the touch, and of brighter yellow-green colour. Ripening proceeded in the L, C, and S treatments at approximately the same rate, except that in L plants the upper leaves (leaves 14, 15, and 16) matured 2 days earlier than in S plants.

(i) *Composition of Uncured Leaves Expressed on an Area Basis*.—Shading significantly increased leaf area relative to the L treatment, but by only 3 per cent. (Table 1): there was no significant increase over the controls. By contrast, dry weight per unit area was greatly affected by treatment. The mean value for L was 20 per cent. greater than for C and that for S was 22 per cent. lower than C. These differences are largely accounted for by the differences in labile hexose carbohydrate per unit area (Table 1). This fraction was 45 per cent. greater in L than in C, and 64 per cent. less in S than in C. The data was also analysed statistically for differences in the linear regression of the variables on leaf position from the fifth to the sixteenth leaf. The regression coefficients show well-defined differences between L and S in the change of dry weight and carbohydrate per unit area with leaf position, the coefficients being positive with L and negative with S. The regression coefficients of leaf area on leaf position over this range of positions is greater with S than L but not significantly. These differential effects may well be a reflection of duration of treatment, for harvesting was such that leaf number and duration were directly related.

Total nitrogen per unit area was little affected by treatment though the values for leaves 15 and 16 were appreciably higher for S than for L and C. Protein nitrogen on this basis was approximately equal in L and S leaves.

Since treatment had such small effects on the areas of individual leaves it will be apparent that leaf composition on an area basis gives a picture of the absolute changes brought about by treatment. Clearly, then, carbohydrates have been increased by extra light and decreased by shade. Nitrogen intake and protein synthesis, on the other hand, have been little affected by treatment.

(ii) *Composition of Uncured Leaves Expressed on a Dry Weight Basis.*—While changes in chemical composition on an area basis have certain advantages in the present case, the dry weight basis is the more usual mode of expression.

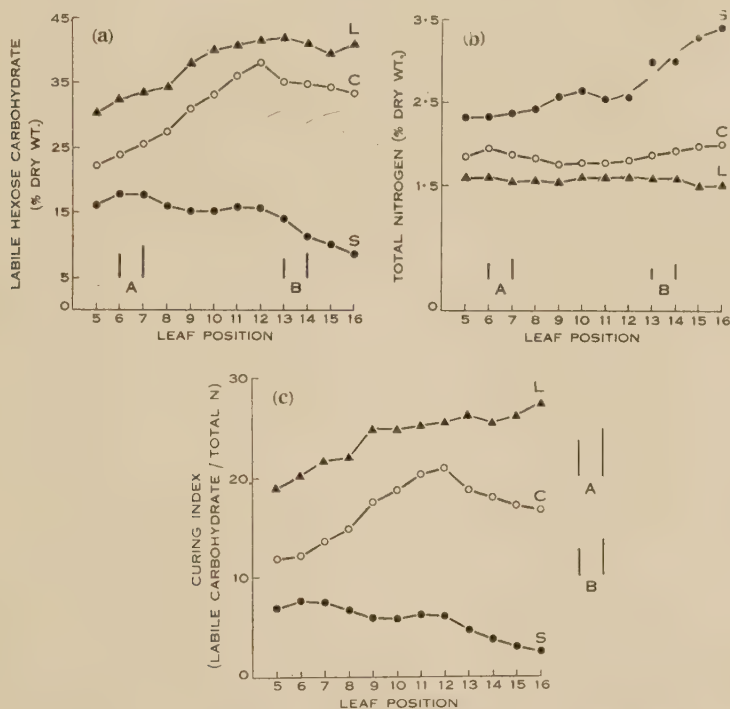


Fig. 1.—Labile hexose carbohydrate (a), total nitrogen (b), and curing index distributions (c) in uncured leaf from extra-light (L), control (C), and shaded (S) plants. Each value is the mean for two rows of plants. A, least difference for significance between treatments within leaf position ($P < 0.05$ and 0.01 respectively). B, least difference for significance between leaf position within treatments ($P < 0.05$ and 0.01 respectively).

The effects of the three treatments on the relative contents of labile hexose carbohydrate and total nitrogen as affected by leaf position are shown by the curves in Figure 1. The ratios of these quantities are also presented as curing coefficients (Johanson 1954*b*, and see below). The leaf from L rows has significantly more carbohydrate (average 38 per cent.) than the leaf from C rows (average 31 per cent.), while S rows produced leaf with only about 15 per cent. of carbohydrate. In L and C plants, the carbohydrate content increases steadily to about position 11 and then stays fairly constant. In contrast to these trends, the leaves of the shaded plants from position 6 to 16 showed a gradual but a highly significant reduction in carbohydrate (Fig. 1).

TABLE I
AREA PER LEAF, AND DRY WEIGHT AND COMPOSITION PER UNIT AREA, IN UNCURED LEAF FROM EXTRA-LIGHT (L), CONTROL (C), AND SHADED (S) PLANTS

Leaf Position No.	Area/Leaf (cm ²)			Dry Weight (mg/cm ²)			Labile Hexose Carbohydrate (mg/cm ²)			Total N (mg/cm ²) (× 100)			Protein N (mg/cm ²) (× 100)	
	L	C	S	L	C	S	L	C	S	L	C	S	L	S
5	862	872	870	4.36	3.83	3.20	1.32	0.85	0.51	7.00	7.14	7.45	4.62	5.56
6	844	870	857	4.51	3.98	3.34	1.46	0.95	0.60	7.28	7.80	7.83	5.23	5.84
7	906	936	939	4.66	4.27	3.16	1.56	1.10	0.56	7.27	8.05	7.54	—	6.00
8	918	949	944	4.65	4.14	3.04	1.60	1.14	0.49	7.41	7.63	7.39	5.30	5.59
9	922	954	949	4.50	3.65	2.93	1.72	1.13	0.45	7.01	6.46	7.55	5.58	—
10	946	957	962	4.76	4.03	3.14	1.91	1.34	0.48	7.70	7.17	8.32	6.44	5.86
11	978	986	1023	4.99	3.99	3.15	2.03	1.44	0.50	8.07	7.10	8.06	—	—
12	983	1002	1034	4.79	3.99	3.18	1.99	1.53	0.50	7.83	7.29	8.23	5.70	6.14
13	901	930	903	5.24	4.11	2.86	2.21	1.45	0.41	8.40	7.73	8.61	—	—
14	987	1011	1018	4.48	3.69	2.70	1.84	1.29	0.31	7.21	7.16	8.14	4.88	5.13
15	895	914	914	4.93	3.98	3.10	1.95	1.37	0.32	7.45	7.94	10.20	—	—
16	930	955	998	5.00	3.89	3.15	2.05	1.31	0.28	7.61	7.98	10.80	5.51	6.11
General mean	923	946	951	4.74	3.96	3.08	1.80	1.24	0.45	7.52*	7.35*	7.91*	—	—
Least difference for significance at $P < 0.05$ between general means	23			0.12			0.13			Not significant				
Regression coefficient (increase per leaf position)	3.32	3.34	4.53	0.024	-0.004	-0.011	0.032	0.023	-0.013	0.046	-0.010	0.041		
Least difference for significance at $P < 0.05$ between coefficients	Not significant			0.008			0.015			Not significant				

* For total N, means include leaves 5-14 only.

Total nitrogen values increased markedly with leaf position in S plants and were about twice as much at high positions as they were in L plants. Total nitrogen content was much lower in the leaves of the control plants and still lower in those of the extra-light plants. In neither of these latter two cases did leaf position have much effect.

The protein nitrogen content of S leaf (Table 2) is significantly higher (by about 60 per cent.) than that of L leaf, but both remain fairly steady with leaf position. In L leaf, non-protein nitrogen values decrease to position 9 and then increase. In S plants these values are fairly uniform to position 12 and then rise steeply (Table 2), and it is this rise which probably explains the increase in total nitrogen in leaves at high positions in S plants (Fig. 1).

TABLE 2

NITROGEN AND RESERVE HEXOSE CARBOHYDRATE* IN UNCURED LEAF FROM EXTRA-LIGHT (L) AND SHADED (S) PLANTS

All figures are percentages of leaf dry weight

Leaf Position No.	Extractables in Leaf		Protein N in Leaf		Non-protein N in Leaf		Reserve Hexose Carbohydrate in Leaf	
	L	S	L	S	L	S	L	S
5	39.3	38.1	1.06	1.74	0.53	0.68	18.8	6.91
6	36.9	41.0	1.16	1.75	0.36	0.62	23.1	8.72
8	37.1	35.7	1.14	1.90	0.29	0.57	24.5	9.73
9	33.5	35.3	1.24	1.84	0.17	0.76	25.0	9.45
10	33.6	36.6	1.21	1.87	0.39	0.78	24.4	10.50
12	32.9	40.5	1.19	1.93	0.40	0.60	24.5	9.35
14	31.8	41.3	1.09	1.90	0.40	1.09	27.9	7.80
16	30.1	41.8	1.10	1.94	0.44	1.53	32.8	5.81

* "Labile hexose carbohydrate" minus "soluble hexose" (see Section II(c)(i)).

The values for extractables (Table 2) indicate that there was a treatment effect only for the higher leaf positions in L plants. Extractables include non-protein nitrogenous compounds together with all other substances removed by 80 per cent. ethanol. The data for reserve carbohydrate suggest that in L leaf with better light conditions greater storage was possible. The reserve carbohydrate of L plants shows relatively small variation in leaf positions 6-12, but values tend to rise thereafter, while in S leaf the trend for reserve carbohydrate is to decrease from leaf 10 towards the top.

(iii) *Comparisons of Cured and Uncured Leaves.*—The quality after curing of the leaves which, uncured, furnished the above analyses, is summarized in Figure 2. The average percentages of good leaf were 94 per cent. in L, 55 per cent. in C, and nil in S. The average percentages of trashy leaf were 86 per cent. for S, 11 per cent. for C, and nil for L. That the proportion of leaf with a green cast was low and fairly

constant was a useful indication of completeness of curing. Furthermore, for individual leaf positions there was a high correlation of the percentages of good cured leaf with the carbohydrate values, and with the ratio of percentages of labile hexose

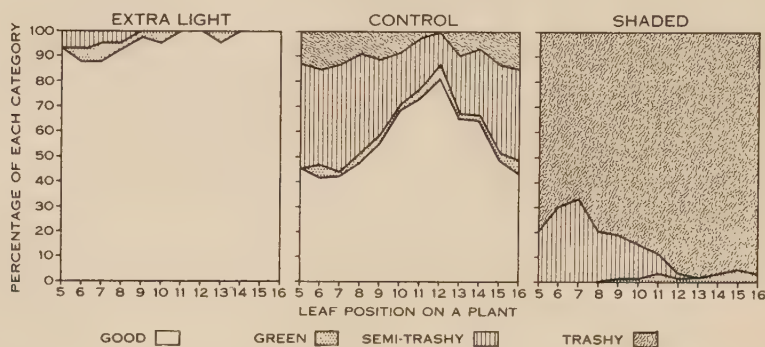


Fig. 2.—Distribution of good, green, semi-trashy, and trashy leaf after curing of leaf from the three treatments.

carbohydrate to total nitrogen in uncured leaf (Fig. 1). From experimental data other than that described in this paper, these ratios (the curing indices) have been found to give a useful measure of the curing potential (cf. Johanson 1954*b*).

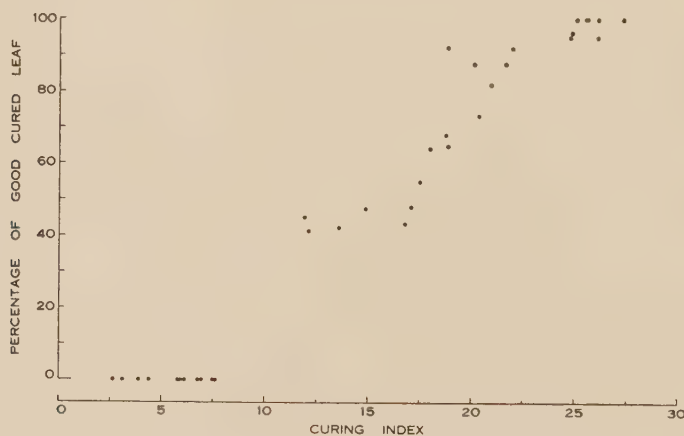


Fig. 3.—The percentage of good cured leaf as a function of the curing indices (derived from analysis of uncured leaf from field experiments).

Figure 3 shows the direct relation between the quality of cured leaf (Fig. 2) and the curing index (Fig. 1). Any crop with a curing index below 15 is unlikely to yield more than 50 per cent. of good leaf.

(b) Effect of Nitrogen Treatment

All experiments so far described were carried out with the samples gathered in Queensland. A further study was made with glass-house-grown material, and besides comparisons before and after curing, a preliminary study of the effect of different levels of nitrogen on curing properties of leaves was included.

TABLE 3

EFFECT OF COMPOSITION ON CURING PROPERTIES OF LEAF FROM GLASS-HOUSE PLANTS
Each value represents a mean composition of eight leaves taken from stalk positions 9 to 16

Treatment	Potential Quality	Labile Hexose Carbo-hydrate (% dry wt.)	Total Nitrogen (% dry wt.)	Curing Index (carbo-hydrate/nitrogen ratio)	Cured Leaf	
					Quality*	Total Good Leaf Area (%)
Experiment 1						
N ₀	Good	38.7	1.45	26.7	Good	90
N ₀	Good	31.2	1.43	21.8	Good	80
N ₁	Indefinite	32.7	2.18	15.0	Fair	50
N ₁	Indefinite	22.8	2.10	10.8	Poor	30
N ₂	Amenenic	16.7	3.38	4.95	Trashy	Nil
N ₂	Amenenic†	11.7	3.75	3.12	Trashy	Nil
Experiment 2						
N ₀	Good	24.9	0.92	27.0	Good	90
N ₁	Indefinite	25.4	1.81	14.6	Poor	40

* Assessed by texture and colour.

† Copious evolution of ammonia gas occurred during curing.

As shown in Table 3, in leaf of about the same carbohydrate status in experiment 1, the treatment N₁ caused an increase of about 52 per cent. in nitrogen; in experiment 2 the nitrogen content was doubled. Increase in nitrogen, however, may be accompanied by an extensive decrease in labile carbohydrate as is strongly evident in treatment N₂. These effects produce a marked reduction in curing indices. Once again there was a good correlation between the curing index and the quality of the cured leaf.

(c) Light Absorption

The prevalence of low light conditions in tobacco stands may be attributed in part to the broadness and overlapping habit of the leaves and, in part, probably more so than in other plants (cf. Seybold and Weissweiler 1942, 1943; Rabideau, French, and Holt 1946), to a considerable capacity of upper leaves to absorb sunlight.

Measurements with a light-meter of the total light transmission through leaves were made by holding the leaf blades against the photocell and exposing their uppersides to vertical solar radiation. The values so obtained were related to full

sunlight as percentage transmission. The values for green, light green, and yellow-green leaves grown in high light intensities in the glass-house were 4.2 ± 0.2 , 6.6 ± 0.2 , and 14.3 ± 0.3 , while the values for comparable leaves from the field were 3.0 ± 0.2 , 6.4 ± 0.2 , and 10.6 ± 0.2 (each value is the mean of eight determinations). It was also found that the percentage-transmission values remain almost unaltered in the range 2000–10,000 f.c. of solar radiation. Thus even at maximal solar intensity a single leaf can reduce the transmitted light to some 300 f.c.

Not only is the light intensity reduced to these extents, but its quality is greatly changed. Partly for this reason the spectrophotometric measurements were made. The curves of Figure 4 show that the transmitted light after passing through a leaf is mainly of a wavelength composition (500–600 $m\mu$) virtually useless for photo-

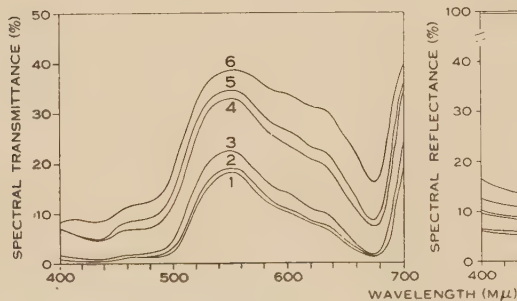


Fig. 4

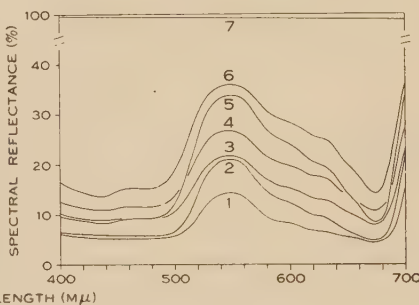


Fig. 5

Fig. 4.—Spectral transmittance of fresh glass-house-grown tobacco leaves. Curves 1, 3, and 5 were obtained for green, light green, and yellow-green leaves respectively grown in high light intensities, and curves 2, 4, and 6 for the corresponding leaves grown in low light intensities. Fig. 5.—Spectral reflectances of fresh glass-house-grown tobacco leaves. Curves 1, 2, and 5 were obtained for the upper side of green, light green, and yellow-green leaves respectively, and curves 3, 4, and 6 are for the underside of the same leaves. Curve 7, standard magnesium carbonate surface. Virtually identical curves were found whether the leaves were grown at 2000 or 6000 f.c.

synthesis. The shape of these curves is due largely to light absorption by chlorophyll, and the very great absorption in the blue and red regions, decreasing with yellowing (loss of chlorophyll), is to be expected. It is of interest that transmission in the green region never exceeds 40 per cent. of the incident light even with yellow leaves.

Incident light which is reflected from leaf to leaf is also of minimal photosynthetic value. Direct measurements (Fig. 5) showed that virtually only green light is reflected. The upper sides of leaves reflected less incident light than the undersides.

IV. DISCUSSION

(a) *The Effect of Composition on Curing Properties of Tobacco Leaf*

The outstanding characteristic of the leaves of flue-cured varieties is their high non-structural carbohydrate fraction, representing in some samples as much as 50 per cent. of the total dry matter (see also Weiss 1950). Because of this, the carbohydrate changes in the tobacco plant as affected by shading are important, as substantial

losses of carbohydrates occur during curing (cf. Ward 1942; Frankenburg 1946; Bacon, Wenger, and Bullock 1951; Askew *et al.* 1954). A further reason for our concentration on this fraction is the fact that in samples with high initial labile-carbohydrate content, other constituents (expressed on original dry weight) do not change markedly during curing (cf. Bacon *et al.* 1951, Askew *et al.* 1954), and the leaf acquires desirable composition. At the same time there is evidence of the importance of the carbohydrate-nitrogen ratio of the fresh leaf (cf. Johanson 1951). This ratio, has been referred to as the curing index; it can vary widely (Fig. 1 and Table 3), and is associated with marked differences in curing properties (cf. Johanson 1954b).

Actually, the effect of carbohydrate and nitrogen is more complex than is simply expressed by ratios of the percentages of these components. From the study of absolute changes during curing (not discussed here) it is evident that both carbohydrate and nitrogen have effect on the loss of dry matter, but nitrogen has the greater effect. However, curing indices are readily determined and they do give a reasonable assessment of the potential quality of uncured leaf, more so than carbohydrate value or nitrogen value alone. The importance of relative proportions of these two components to each other is illustrated by the data presented in Table 3. Here, in the leaves designated "indefinite", the percentages of carbohydrate are comparatively high, yet the quality of the cured product is unsatisfactory. An extreme contrast is that of experiment 2, where there are two samples of leaf with similar carbohydrate values but high and low nitrogen contents. Richards (1938), Petrie and Williams (1938), and others have demonstrated a correlation between protein nitrogen and respiratory activity in plants, and this may provide a partial explanation of the dependence of curing potential upon the balance between carbohydrate and nitrogen in the leaf.

Leaves with high curing indices retain their yellow pigments and fluorescence after normal heat-curing; in amenenic leaves these components are lost and, in addition, ammonia may be liberated at an early stage of curing. In our experiments it was found that while amenenic leaf may reach the stage of evolution of ammonia within 48 hr, high-carbohydrate leaf would require as much as 200 and more hours to reach this stage. It seems clear that in low-carbohydrate (amenenic) leaf no sparing of yellow pigments and other desirable compounds, and proteins, occurs and leaf is forced to metabolize these under starvation conditions of curing. Such influence of initial composition on the metabolism of detached leaves is widely recognized. Yemm (1935, 1937), working with barley leaves, found in starvation a similar sparing action of carbohydrates, and a similar eventual utilization of other substrates, with final browning and evolution of ammonia. We have found (unpublished data) a similar pattern with tomato (*Lycopersicon esculentum* Mill.) and with egg-plant (*Solanum melongena* L.)

(b) Effect of Shading the Tobacco Plant on the Properties of Its Leaves

The physical characteristics of green leaves with a deficiency of carbohydrate relative to nitrogen have been described by Kraus and Kraybill (1918) who found succulent, thin-walled cells, with soft texture—a similar pattern to that found in our shaded tobacco plants. Besides these characteristics, and lack of fluorescence

(Johanson 1953), the marked difference in shaded tobacco leaves is the change in composition. There was a decrease in carbohydrate content for all leaf positions as shading increased ($L > C > S$) whether the analyses were expressed on a basis of dry weight (Fig. 1) or of leaf area (Table 1). Furthermore, for the S leaves, the amount of decrease became progressively greater with duration of shading. However, the increase in carbohydrate contents of L leaves relative to controls did not alter with time. The distribution of carbohydrate with leaf position on the plants in the L and C treatments closely followed the usual pattern (cf. Askew *et al.* 1948; Darkis *et al.* 1952). For total nitrogen, there was a general increase in all leaf positions in analyses related to dry weight as shading increased (Fig. 1), but on a leaf-area basis there was no significant difference between treatments of either total nitrogen or protein nitrogen.

The mechanism of the fall in carbohydrates in our leaves as light intensity during the day was decreased is probably qualitatively similar to that underlying the diurnal variations in tobacco-leaf carbohydrates, described by Moroz-Morozenko (1935). Apparently our shaded plants were not able to accumulate sufficient carbohydrate by day to do much more than compensate for the losses by night. The rapidity of the change in carbohydrate was striking; the changes in bottom leaves shown in Table 1 resulted from only 4 days artificial shading. On the other hand, the lack of change of nitrogen on an area basis is consistent with the evidence for a sparing action of carbohydrate on protein in starving plants and detached leaves (cf. Mothes 1925; Paech 1935; Kabos 1936; Vickery *et al.* 1937, 1939; Wood, Cruickshank, and Kuchel 1943).

(c) *Shading in the Field*

It is reasonable to infer from the carbohydrate data of this experiment that serious interference with photosynthesis may occur because of self-shading. This factor is likely to be more important for tobacco than for other crops such as corn (Verduin and Loomis 1944), where it has been shown that 30–40 per cent. of full sunlight penetrates to the lower leaves. The light that penetrates to the lower leaves of a well-grown stand of tobacco is not only reduced in quantity but also in quality, for the absorption spectra of Figures 4 and 5 are strikingly similar to those of purified chlorophylls *a* and *b* (Zscheile and Comar 1941).

The artificial shade of the Queensland experiment reduced the light intensity to about 1000 f.c., but the rapid deteriorations of these shaded leaves may be attributed to further internal shading by the leaves themselves. Heinicke and Childers (1937), Thomas and Hill (1937), and Kramer and Clark (1947) have shown for other plants that internal shading is affected by the intensity of the outside light, and Thomas and Hill (1937) have shown that light intensities on cloudy days can themselves limit the rate of photosynthesis. Since north Queensland is occasionally subject to periods of cloudy weather it is quite possible that this factor alone was responsible for the large amounts of trashy leaf produced during the rainy weather in 1933 (Tobacco Inquiry Committee 1933) and in 1950. In 1954 at Tenterfield, N.S.W. (29°S.), a large proportion of trashy leaf was produced during persistent cloudiness, and good quality leaf after the weather cleared (see Table 4).

(d) Sunlight at Four Latitudes

It is of interest to compare the duration of light periods at four latitudes in the main tobacco areas of eastern Australia. The hours of sunshine given in Table 5 are aggregates for 120 days representative of the growing seasons at the latitudes

TABLE 4

CARBOHYDRATE AND NITROGEN CONTENT OF TRASHY LEAF FROM TENTERFIELD, N.S.W., HARVESTED DURING CLOUDY AND RAINY WEATHER, FEBRUARY 1954

Sample*	Description	Labile Hexose Carbohydrate (% dry wt.)	Total N (% dry wt.)
29-1 (cutters)	Trashy. Few small faintly yellow areas, thin texture	9.23	2.14
29-2 (cutters)	Trashy. Complete absence of yellow, thin texture	4.67	2.60
29-3 (leaf)	Trashy. Complete absence of yellow, fairly thick texture	7.47	3.12
29-4† (cutters)	Good leaf. All yellow, medium texture	31.3	1.84

* Samples 29-1 and 29-2 are from the same commercial crop. Samples 29-3 and 29-4 are from the same experiment station site.

† Sample 29-4 was harvested after the weather cleared.

indicated, and are based on the data supplied by the Commonwealth Meteorological Bureau, Melbourne. Thus there are 12.2, 17.4, and 22.4 per cent. more meteorological hours of sunshine available at 19, 29, and 36°S. respectively than at 17°S. latitude. However, the true photosynthetic (physiological) hours will lie between the values for astronomical and meteorological hours of sunshine, and may be computed from the average duration of cloudy periods for each area (see Table 5). On this basis 9.64, 15.4, and 20.4 per cent. more light-hours respectively are available at the higher latitudes than at 17°S.

Since the formation of photosynthetic products occurs in the light and only their consumption in the dark, then, other factors being equal, areas with longer dark periods are at a distinct disadvantage. The percentage decrease in the dark period relative to the amount at 17°S. is given in the last column of Table 5. For the extreme comparison of 36°S. with 17°S. the dark period is 12 per cent. less and photosynthetic hours of sunshine are 20 per cent. greater for the higher latitude. Actually these differences are larger, as the growing season in the south could be as much as 4 or more weeks longer. Thus the probable effect of the longer photosynthetic hours could favour higher carbohydrate status of tobacco plants, per any unit time, and any short period reduction of sunlight would be less detrimental to the quality of leaf than it would be in areas of shorter daylight.

In this paper it has been shown that artificial reduction in light intensity, under field conditions, before harvesting, adversely affects composition and favours development of amenenic leaf. In commercial crops, adverse light conditions would

occur at intervals throughout the whole growing period. When they occur during the harvesting period, and are as severe as those produced by the artificial shading, such conditions could almost wholly account for loss of the crop. It is probable that light is often the limiting factor in the carbohydrate economy of tobacco plants. The

TABLE 5
TOTAL HOURS OF SUNSHINE AND DARKNESS AT LATITUDES 17, 19, 29, AND 36°S. AVAILABLE TO
TOBACCO CROPS DURING 120 DAYS GROWING PERIOD*

Latitude	Hours of Sunshine			Hours of Physio- logical Dark Period	Percentage Difference in Physiological Light and Dark Period Relative to 17°S.	
	Astro- nomical	Meteoro- logical	Physio- logical†		Increase in Light	Decrease in Dark
17°S.	1482	980	1080	1800	—	—
19°S.	1521	1100	1184	1696	9.64	5.78
29°S.	1633	1150	1246	1634	15.4	9.23
36°S.	1698	1200	1300	1580	20.4	12.2

* Growing periods for 17 and 19°S. latitudes taken as from September 3 to December 31 and for 29 and 36°S. latitudes as from November 1 to February 28.

† It was assumed that 5 hr of overcast sunlight was photosynthetically equivalent to only 1 hr of bright sunlight. A fifth of the difference between astronomical and meteorological hours of sunshine was added to the meteorological hours of sunshine in each case.

adverse effects of light limitation would be greatest during periods of persistent cloudiness or where conditions favour unrestricted growth and therefore internal shading.

V. ACKNOWLEDGMENTS

Grateful acknowledgment is made to Mr. R. F. Williams, Division of Plant Industry, C.S.I.R.O., for his constructive criticism of the manuscript, and to Mr. G. A. McIntyre, Division of Mathematical Statistics, C.S.I.R.O., for statistical analysis of the data. Special thanks are due to Mr. K. A. Wright, Division of Physics, C.S.I.R.O., for making available a G. E. spectrophotometer and for providing spectral graphs; and to Mr. L. J. Dwyer, Director, Meteorological Branch, Department of Interior, Melbourne, for supplying the data which constitute the basis for Table 5.

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THE EFFECT OF MINERAL NUTRITION ON THE CONTENT OF FREE AMINO ACIDS AND AMIDES IN TOMATO PLANTS

II. A STUDY OF THE EFFECT OF MOLYBDENUM NUTRITION

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[*Manuscript received July 19, 1956*]

Summary

Tomato plants which had been cultured in the absence of molybdenum were provided with molybdate, and the consequent changes in the relative amounts of the free amino acids and amides were followed over an experimental period of 4 days. The technique of quantitative paper chromatography was used to estimate the individual free amino acids and amides, and the results are expressed on a dry weight basis.

Changes in the concentrations of many of the individual amino acids occurred within 4 hr of applying molybdenum. Large increases in the concentration of aspartic acid, glutamic acid, glycine, glutamine, and asparagine occurred initially with molybdenum treatment, but were followed by steady declines. A different pattern of change was shown by the two compounds β -alanine and γ -aminobutyric acid, both of which decreased in concentration soon after molybdenum was applied.

The results of this investigation are discussed in relation to the pathway by which the products of nitrate reduction are incorporated into proteins.

I. INTRODUCTION

Spencer and Wood (1954) have shown that the provision of molybdenum to plants deficient in this element promotes rapid changes in the concentrations of a variety of nitrogenous compounds. In terms of concentration, nitrate nitrogen decreases and nitrite, ammonia, amino acid and amide nitrogen fluctuate during a 4-day period after treatment. The present investigation has been designed to amplify that of Spencer and Wood with respect to the amino acid and amide fractions. The same general experimental approach has been used, but whereas in the earlier investigation only total amino nitrogen and total amide nitrogen were measured, in this an attempt has been made to observe changes with individual compounds.

Tomato plants cultured in the absence of molybdenum were provided with molybdate, and the consequent changes in the concentrations of the individual amino acids and amides were measured. In the first phase of the investigation an experimental period of 4 days was adopted. The results of this experiment, however, showed that important changes occurred during the first 24 hr after the addition of molybdenum, and in a later phase, therefore, an experimental period of less than 24 hr was incorporated in the design with frequent samples during this period.

This investigation, like that of Spencer and Wood, is intended as a contribution to the elucidation of the nutritional effect of molybdenum. It may be noted, however,

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that since Nicholas, Nason, and McElroy (1953) have shown that molybdenum is required for the activity of nitrate reductase, the present series of observations are also of some significance in relation to the mechanism of nitrate incorporation.

II. METHODS

The culture of tomato plants deficient in molybdenum, and the analysis of extracts of the experimental plants for free amino acids and amides were carried out as described by Possingham (1956). In this investigation the plants were grown in an unheated glass-house and they were kept in the glass-house throughout the experimental period.

III. EXPERIMENTAL AND RESULTS

As indicated above the investigation involved two experimental series. The details of the experimental design and the results are given separately for each series.

(a) *Experiment I*

Twenty-seven pots, each of which held eight molybdenum-deficient plants, provided the material for this experiment. The experimental period commenced when the plants first clearly showed the symptoms of molybdenum deficiency. This was 2 weeks after the seedlings were transferred to culture solutions deficient in molybdenum, and 5 weeks from the commencement of germination.

The experiment involved an unreplicated design in which the set of deficient seedlings was divided into two groups. One group, the untreated series, was not treated further, but to the second, the treated series, a solution of sodium molybdate was added at the commencement of the experimental period. The volume added to each 3-l. pot gave 0.0151 g of the salt per pot which was equivalent to a molybdenum concentration of 2 p.p.m. A random sample of three pots was taken at the beginning of the experimental period, and this is termed the zero time harvest. Subsequently random samples of three pots were taken from each series at intervals of 24 hr over the next 4 days. The sample of three pots comprised 24 plants, and the shoots were removed by cutting through the stem immediately above the cotyledons. From each bulked sample of 24 shoots, six were taken for the determination of dry weights, the remaining 18 shoots being used for the determination of amino acids and amides.

In this and in the subsequent experiment the results of the amino acid analyses are expressed as concentrations in μg of amino acid or amide per mg dry weight. The results for experiment I are given in Table 1, from which it is evident that molybdenum treatment promoted considerable changes.

Statistical assessment of the significance of the many concentration drifts required logarithmic transformation of the values. The data were first examined to ascertain whether the observed increases in the individual amino acid and amide concentrations after molybdenum treatment were contributed to significantly by downward time drifts in the untreated series. Serine, alone of all the constituents, showed a significant downward trend between zero time and 96 hr. Although there is some indication that this is true for other constituents, the trends are erratic in the untreated control plants. Accordingly the five observations on the untreated

TABLE 1

EXPERIMENT I: CHANGES IN DRY WEIGHT (MG) AND CONCENTRATION OF AMINO ACIDS AND AMIDES (μG PER MG DRY WEIGHT) OF MOLYBDENUM-DEFICIENT PLANTS FOLLOWING TREATMENT WITH MOLYBDATE

Amino Acid or Amide	Treatment	Length of Time after Molybdate Addition (hr)				
		Zero Time	24	48	72	96
Aspartic acid	—Mo	0.170	0.234	0.098	0.147	0.114
	+Mo		2.931	1.989	1.827	1.363
Glutamic acid	—Mo	2.950	2.518	3.076	2.083	1.856
	+Mo		12.789	9.185	7.824	5.069
Asparagine	—Mo	0.108	0.144	0.108	0.154	0.292
	+Mo		0.987	1.239	0.662	0.606
Glutamine	—Mo	0.309	0.248	0.160	0.143	0.671
	+Mo		1.852	1.743	3.067	2.120
Citrulline	—Mo	0.295	0.238	0.155	0.132	0.447
	+Mo		0.539	0.287	0.256	0.236
Arginine	—Mo	0.721	0.646	0.222	0.268	0.368
	+Mo		0.559	0.407	0.150	0.101
"Under arginine"*	—Mo	0.515	0.298	0.155	0.222	0.251
	+Mo		0.809	0.297	0.022	0.101
Serine	—Mo	2.548	1.490	1.301	1.138	0.825
	+Mo		2.577	1.932	1.489	1.514
Glycine	—Mo	0.319	0.189	0.129	0.114	0.206
	+Mo		1.617	1.843	0.800	0.943
Threonine	—Mo	0.273	0.323	0.279	0.199	0.206
	+Mo		0.770	0.687	0.645	0.808
α -Alanine	—Mo	1.014	1.082	0.806	0.934	0.591
	+Mo		2.003	1.656	1.022	1.649
β -Alanine	—Mo	0.113	0.174	0.155	0.154	0.109
	+Mo		Absent	Absent	Absent	Absent
γ -Aminobutyric acid	—Mo	0.515	1.058	1.094	1.300	0.653
	+Mo		Absent	Absent	0.022	0.303
Ethanolamine	—Mo	0.257	0.263	0.222	0.137	0.251
	+Mo		0.086	0.074	0.044	0.075
Phenylalanine	—Mo	0.196	0.234	0.186	0.069	0.206
	+Mo		0.068	0.091	0.130	0.124
Valine	—Mo	0.294	0.308	0.114	0.154	0.150
	+Mo		0.077	0.062	0.133	0.163
Leucine	—Mo	0.232	0.462	0.186	0.205	0.251
	+Mo		0.135	0.142	0.145	0.172
Proline	—Mo	0.772	0.845	1.363	0.821	0.413
	+Mo		1.348	1.411	1.022	0.959
Total amino acids	—Mo	12.714	10.670	9.541	8.077	6.897
	+Mo		26.374	20.034	15.531	13.580
Total amides	—Mo	0.417	0.392	0.269	0.297	0.963
	+Mo		2.839	2.982	3.729	2.726
Dry weight per plant (mg)	—Mo	10.79	14.38	20.77	20.38	23.06
	+Mo		14.42	22.43	24.99	33.00

* See Part I of this series (Possingham 1956).

plants were used to estimate the variance of the mean of the untreated series, against which the various molybdenum treatments were compared. As only a single observation could be made at each harvest, owing to the complexity of amino acid analysis, it was necessary to assume that the variance of the untreated series would also apply at each harvest of the treated series. Thus the significance of the changes in each constituent after 24 hr molybdenum treatment was assessed. In addition an assessment of the changes in each constituent of the molybdenum-treated plants

TABLE 2

EXPERIMENT I: STATISTICAL SUMMARY OF THE RESULTS

The results are the transformed values of the concentrations of amino acids and amides (transformation $\log(1000x)$)†

Amino Acid or Amide	—Mo (mean of —Mo treatments)	+Mo (24 hr)	Difference	Regression (+Mo 24 hr to +Mo 96 hr)
Aspartic acid	2.163	3.467	1.304***	—*
Glutamic acid	3.389	4.106	0.717***	—*
Asparagine	2.175	2.994	0.819**	—*
Glutamine	2.414	3.268	0.854**	+*
Citrulline	2.362	2.732	0.370	—
Arginine	2.602	2.747	0.145	—*
“Under arginine”	2.424	2.908	0.484	—
Serine	3.133	3.411	0.278*	—
Glycine	2.252	3.209	0.957**	—
Threonine	2.401	2.886	0.485**	+
α -Alanine	2.938	3.302	0.364	—
β -Alanine	2.142	Absent	—()***	
γ -Aminobutyric acid	2.941	Absent	—()***	+***
Ethanolamine	2.343	1.934	—0.409*	—
Phenylalanine	2.217	1.833	—0.384*	+
Valine	2.276	1.886	—0.390	+
Leucine	2.402	2.130	—0.272*	+
Proline	2.896	3.130	+0.234	—

* Significant at $P < 0.05$. ** Significant at $P < 0.01$. *** Significant at $P < 0.001$.

† In this table, as also in Table 4, the estimations of amino acid content, reported here to three or four decimal places for convenience of statistical analysis, are probably reliable to within 1 per cent. The statistical treatment of the data has been explained on pp. 41 and 46 of this paper.

over the period from 24 to 96 hr was made by extracting the regressions on time. A summary of these changes with the statistical significance of each is given in Table 2.

It is evident from Tables 1 and 2 that molybdenum treatment enhanced dry weight and increased the concentrations of the amino acid and amide fractions. The total amino acid fraction reached a peak value 24 hr and the amide fraction 72 hr after the beginning of the experimental period.

TABLE 3

EXPERIMENT II: CHANGES IN DRY WEIGHT (MG) AND CONCENTRATION OF AMINO ACIDS AND AMIDES (μG PER MG DRY WEIGHT) OF MOLYBDENUM-DEFICIENT PLANTS FOLLOWING TREATMENT WITH MOLYBDATE

Amino Acid or Amide	Treatment	Length of Time after Molybdate Addition (hr)					
		Zero Time	$\frac{1}{2}$	1	2	4	8
Aspartic acid	—Mo	0.154				0.085	0.099
	+Mo		0.127	0.098	0.393	1.388	2.240
Glutamic acid	—Mo	2.761				1.862	1.987
	+Mo		2.200	1.903	3.037	5.111	6.505
Asparagine	—Mo	0.136				0.102	0.084
	+Mo		0.154	0.121	0.201	0.463	0.697
Glutamine	—Mo	0.289				0.187	0.265
	+Mo		0.260	0.195	0.262	1.545	5.089
Citrulline	—Mo	0.234				0.136	0.084
	+Mo		0.232	0.144	0.178	0.296	0.331
Arginine	—Mo	0.318				0.267	0.298
	+Mo		0.382	0.334	0.277	0.414	0.408
"Under arginine"	—Mo	0.222				0.183	0.091
	+Mo		0.247	0.196	0.174	0.310	0.298
Serine	—Mo	1.147				0.964	1.065
	+Mo		0.920	0.848	1.337	1.873	2.618
Glycine	—Mo	0.121				0.082	0.104
	+Mo		0.116	0.105	0.147	0.293	0.498
Threonine	—Mo	0.208				0.144	0.191
	+Mo		0.233	0.165	0.230	0.358	0.448
α -Alanine	—Mo	0.865				0.569	0.755
	+Mo		0.577	0.687	0.841	1.199	1.693
β -Alanine	—Mo	0.104				0.056	0.086
	+Mo		0.108	0.087	0.056	Absent	Absent
γ -Aminobutyric acid	—Mo	0.995				0.773	0.844
	+Mo		0.871	0.724	0.663	0.310	0.212
Ethanolamine	—Mo	0.212				0.103	0.184
	+Mo		0.275	0.158	0.211	0.099	0.187
Phenylalanine	—Mo	0.172				0.082	0.098
	+Mo		0.163	0.102	0.082	0.103	0.074
Valine	—Mo	0.201				0.159	0.197
	+Mo		0.289	0.152	0.303	0.154	0.105
Leucine	—Mo	0.310				0.188	0.218
	+Mo		0.298	0.312	0.222	0.268	0.122
Proline	—Mo	1.040				0.774	0.924
	+Mo		0.732	0.696	1.152	1.032	1.244
Total amino acids	—Mo	9.064				6.427	7.225
	+Mo		7.770	6.681	9.303	13.208	16.983
Total amides	—Mo	0.425				0.289	0.349
	+Mo		0.414	0.316	0.463	2.008	5.786
Dry weight per plant (mg)	—Mo	31.98				36.62	37.74
	+Mo		36.45	38.32	31.83	39.62	33.48

With respect to the individual constituents, the largest changes were recorded with aspartic acid, glutamic acid, and glycine. The maximum concentrations of both aspartic and glutamic acid were found at 24 hr, when in the treated plants the concentration of aspartic acid was 10 times greater, and that of glutamic acid five times greater, than it was in the untreated plants. After 24 hr the concentrations of both these acids in the treated plants decreased steadily with time. Glycine was at a maximum at 48 hr, when its concentration in the treated plants was approximately 10 times that of the untreated plants. Thereafter this compound also decreased in concentration.

Serine and γ -alanine showed similar although smaller changes, and with both these compounds the highest concentrations were found at 24 hr. Threonine increased rapidly in the first 24 hr and continued to increase slightly over the succeeding 72 hr. β -alanine and γ -aminobutyric acid, although they were present in the untreated molybdenum-deficient plants, disappeared during the first 24 hr after treatment. Although γ -aminobutyric acid was detected in the later phases of the experimental period in the treated plants, β -alanine did not reappear and this substance may therefore be taken as characteristic of the deficient tissue. Ethanolamine, phenylalanine, and leucine decreased as a result of treatment during the first 24 hr. During the final 72 hr ethanolamine continued to decrease while phenylalanine and leucine tended to increase.

With respect to the amides asparagine and glutamine the concentrations of both at first increased and then decreased after treatment. In the case of asparagine a peak value was reached at 48 hr and with glutamine at 72 hr.

(b) Experiment II

Sixteen pots of molybdenum-deficient tomato plants were used in this experiment. The plants were harvested 3 weeks after planting out the seedlings and 6 weeks from the commencement of germination. This experiment was similar to experiment I; it involved an unreplicated design in which the deficient seedlings were divided into two groups. One group was not treated further, but sodium molybdate was added to the second group at the beginning of the experimental period. The untreated group was sampled at the start of the experimental period (zero time) and after 4 and 8 hr. The treated group was sampled at $\frac{1}{2}$, 1, 2, 4, and 8 hr after zero time. Each sample involved two pots or 16 shoots and, as before, separate samples were taken for determination of dry weight and for the determination of amino acids.

The results of both the amino acid analyses and the dry weight estimations are given in Table 3. The dry weight yields of the plants used in this experiment were higher than those recorded in experiment I. The differences were not large and were a result of the greater age of the experimental plants. Approximately the same range and concentrations of amino acids and amides were found in untreated plants at zero time in this experiment as in experiment I.

For statistical analysis the actual concentration values were transformed to logarithms. A summary of this analysis is given in Table 4.

There is a similar difficulty here as in the previous experiment in attempting to form an estimate of error. It was assumed that the variation between zero time, 4, and 8 hr in the absence of molybdenum, and the $\frac{1}{2}$ - and 1-hr treatments with molybdenum, could be ascribed to chance variation between groups of plants. This assumption would tend to give an estimate of variance in excess of the replicate variation if in fact there are real differences between these increases. It was necessary to assume that this variance would also apply to the constituents present at 8 hr in the molybdenum-treated plants.

TABLE 4

EXPERIMENT II: STATISTICAL SUMMARY OF THE RESULTS

The results are the transformed values of the concentrations of amino acids or amides (transformation $\log (1000x)$)

Amino Acid or Amide	-Mo (mean of 5 values)†	+Mo (8 hr)	Difference
Aspartic acid	2.0416	3.350	1.3084***
Glutamic acid	3.3260	3.813	0.4870**
Asparagine	2.0676	2.843	0.7754**
Glutamine	2.3722	3.707	1.3348***
Citrulline	2.2200	2.520	0.3000
Arginine	2.5018	2.611	0.1092
"Under arginine"	2.2504	2.474	0.2236
Serine	2.9952	3.418	0.4230***
Glycine	2.0198	2.697	0.6772***
Threonine	2.2682	2.651	0.3828*
α -Alanine	2.8336	3.229	0.3954**
β -Alanine	1.9344	Absent	-()***
γ -Aminobutyric acid	2.9224	2.326	-0.5964***
Ethanolamine	2.2484	2.272	0.0236
Phenylalanine	2.0724	1.869	-0.2034
Valine	2.2882	2.021	-0.2861
Leucine	2.4142	2.086	-0.4056*
Proline	2.9160	3.095	0.1790

* Significant at $P < 0.05$.

** Significant at $P < 0.01$.

*** Significant at $P < 0.001$.

† Mean of the three -Mo harvests plus the $\frac{1}{2}$ - and 1-hr harvests of the molybdenum-treated series.

In the comparison between the untreated plants and those treated with molybdenum for 8 hr the important group of compounds aspartic acid, glutamic acid, asparagine, and glutamine all showed significant increases in concentration in the treated plants. The changes in the cases of aspartic acid, glutamic acid, and asparagine had begun probably between 1 and 2 hr after the molybdenum was applied while the increase in glutamine concentration was initiated between 2 and 4 hr after zero time. Serine, glycine, threonine and α -alanine all increased in concentration in the molybdenum-treated plants. The changes started between 1 and 2 hr after treatment in the cases of serine and glycine and between 2 and 4 hr with threonine and α -alanine.

Three other significant changes had taken place in 8 hr in the molybdenum-treated plants. They were all decreases in concentration and involved the compounds β -alanine, γ -aminobutyric acid, and leucine. The concentration of β -alanine decreased between 2 and 4 hr to the extent that this constituent was not detectable in the 4-hr sample of the treated plants. The downward trend in the concentration of γ -aminobutyric acid commenced between 2 and 4 hr, while that of leucine started between 4 and 8 hr after treatment.

IV. DISCUSSION

The present series of observations are restricted to the effects of molybdenum on the amino acids and amides. It is clear however that the significance of these effects can be evaluated only by reference to the accompanying changes in the nitrate and protein fractions. Measurements of these have been made by Spencer and Wood (1954) whose results are immediately comparable with those of this investigation since they were made with a basically similar experimental approach.

Changes in the amino acid and amide fraction may be considered in terms of a system in which nitrate is reduced to ammonia, the ammonia reacts with a keto acid to give an amino acid, and this in turn is finally incorporated into a protein. In this sequence the position may be complicated by transamination reactions in which the products of nitrate reduction are transferred from one carbon skeleton to another, and by amidations in which ammonia combines with a dicarboxylic acid to give an amide. These, however, do not affect the situation that a basic sequence is probably involved in which the concentrations of the amino acids considered as intermediates are likely to be determined by the rate of nitrate reduction, on the one hand, and by the rate of incorporation into protein on the other, but modified by transamination rates.

Nicholas, Nason, and McElroy (1953) have shown that molybdenum is required in the nitrate reductase system. Spencer and Wood (1954) have shown that nitrate accumulates in the absence of molybdenum. These workers, however, have also shown that, when molybdenum is added, nitrate is reduced with a consequent increase in total amino and amide nitrogen. In experiments I and II of this investigation an increase in total amino acids and amides has also been demonstrated. In experiment II the increase occurred within 8 hr, and, during this time, it is unlikely that a large quantity of protein was synthesized, and the data therefore suggest that the initial accumulation of amino acids and amides results from the rapid acceleration in the reduction of nitrate.

During this first phase of increase in the total amino acid level, it is significant that the greatest increases are recorded with aspartic and glutamic acids and with glycine. The rapid increase in the dicarboxylic acids is no doubt a consequence of the reaction of ammonia with oxaloacetic and α -ketoglutaric acids; and in this connection it is significant that Vickery *et al.* (1940) have shown that when ammonia carrying isotopic nitrogen is fed to tobacco plants the most rapid and extensive incorporation is observed with glutamic and aspartic acids.

The significance of the rapid increase in glycine cannot be evaluated from the present series of data. It is known that, in the animal body, glycine may be

formed by a degradation of serine (Shemin 1946); but the data of experiment II do not indicate a decrease in serine corresponding to an increase in glycine. Weinhouse (1955) has shown that glycine can also be formed in transamination reactions involving glyoxylic acid and a whole range of amino acids. It is possible that in molybdenum-deficient plants an accumulation of glyoxylic acid, or its precursor, glycolic acid, may occur, and this may be the basis of the rapid increase in this amino acid. For this interpretation however, no evidence is available.

Several amino acids increased slightly during the initial phase, and these increases are no doubt due to secondary reactions consequent on the formation of glutamic and aspartic acids.

Some amino acids decrease in concentration from the beginning of the experimental period and most tend to decrease during the later phases. The data of Spencer and Wood (1954) indicate that the decreases occur during a phase of protein accumulation, and many of the decreases must be attributed to incorporation into protein. This is probably also the reason for the final decrease in amide concentration, since Damoradan, Joaback, and Chibnall (1932) and Damoradan (1932) have shown that asparagine and glutamine are constituents of some plant proteins.

Some of the decreases may be due to other reactions. The dicarboxylic amino acids decrease after the initial rise during the first 24 hr. It is significant, however, that the concentration of the amides continues to increase. This suggests that at least part of the decrease in the concentration of glutamic and aspartic acids is due to a conversion of these compounds to the corresponding amides by reactions such as those described by Elliott (1951) and by Webster (1955).

The decreases in the concentrations of β -alanine and γ -aminobutyric acid are also probably not due to direct incorporation. Steward, Thompson, and Dent (1949) have recorded a similar dispersal of one of these compounds, γ -aminobutyric acid, during a phase of rapid protein synthesis, but it is significant that neither of these compounds has been identified in protein hydrolysates. It may be that they are consumed in reactions that accompany protein synthesis, since Miettinen and Virtanen (1953) have shown that γ -aminobutyric acid can transaminate with α -ketoglutarate, and Steward and Pollard (1956) have provided evidence showing that γ -aminobutyric acid may be readily converted to glutamic acid.

V. ACKNOWLEDGMENTS

The author is indebted to Mr. G. A. McIntyre, Division of Mathematical Statistics, C.S.I.R.O., for statistical treatment of the data.

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THE EFFECT OF SULPHUR DEFICIENCY ON THE FREE AMINO ACIDS OF SOME PLANTS

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[Manuscript received August 10, 1956]

Summary

White clover (*Trifolium repens* L.) and four other species were grown in water culture with and without sulphur, and a semi-quantitative comparison was made of the free amino acid contents, using paper chromatography.

Free arginine occurs in greatly increased amounts in sulphur-deficient *Desmodium uncinatum* DC., white clover, tomato, and flax and, with the exception of flax, the glutamic acid content of these plants is much reduced and some amide accumulation occurs. Asparagine is the predominant accumulating amide in the sulphur-deficient leguminous species, while in tomato glutamine predominates; barley contains about equal amounts of both. The amide present in greatest amount in the deficient plants is that which occurred in greatest amount in the normal plants. The amino acids glycine and serine increase in all the above species where sulphur is in short supply.

Citrulline, which has not been previously found in flax, occurs in moderate amount in the sulphur-deficient plants.

I. INTRODUCTION

It is well known that sulphur deficiency results in profound changes in the nitrogen metabolism of plants. Protein synthesis is checked and soluble organic and inorganic nitrogenous compounds accumulate. Nightingale, Schermerhorn, and Robbins (1932) found a greater amount of free amino and amide nitrogen in sulphur-deficient tomatoes than in the normal plants. Similar results were reported by Eaton (1935, 1941, 1942) in soybean, sunflower, and mustard, and by Anderson and Spencer (1950) in flax, oats, and subterranean clover plants. Mertz, Singleton, and Garey (1952) measured, microbiologically, the arginine, aspartic acid, glutamic acid, and methionine contents of hydrolysed dialysates of the leaves and stems of alfalfa of both the normal and sulphur-deficient plants, and found greater amounts of arginine and aspartic acid in the hydrolysates of the deficient plants. No distinction was made between the amount of free aspartic acid in the plants and that derived from asparagine by hydrolysis. In the present experiments, paper chromatography has been used as a semi-quantitative method to examine the free amino acid contents of several plant species as affected by sulphur deficiency.

II. EXPERIMENTAL

(a) Cultural Method

White clover (*Trifolium repens* L.) var. Ladino, the legume *Desmodium uncinatum* DC., flax (*Linum usitatissimum* L.), tomato (*Lycopersicon esculentum* Mill.) var. Grosse Lisse, and barley (*Hordeum vulgare* L.) var. Spratt Archer were grown in water culture with and without added sulphate. The white clover, tomato,

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flax, and barley plants were grown in the months of July, August, and September of 1954 and 1955, and *D. uncinatum* in February and March 1956. After germination in vermiculite the seedlings were transferred to nutrient tanks. The nutrient solutions contained the following salts in the number of milligrams per litre shown: $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (945); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (407); $\text{NH}_4\text{H}_2\text{PO}_4$ (115); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (3.55); $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.06); ZnCl_2 (0.10); H_3BO_3 (1.43); $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ (85 per cent. MoO_3) (0.09). Potassium, sulphur, and additional nitrogen were given to the normal plants as K_2SO_4 (521 mg/l) and NH_4NO_3 (240 mg/l). The sulphur-starved plants were given potassium and extra nitrogen as KNO_3 (607 mg/l). Iron was added as required (0.6 ml/l of a solution containing 0.36 per cent. $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 0.4 per cent. tartaric acid added two or three times weekly). The nutrient solutions were renewed at intervals of 14 days.

(b) *External Effects of Sulphur Deficiency*

Symptoms of deficiency were evident in all species 4–5 weeks after germination, when the young leaves of the deficient plants were a yellowish green colour. Successive leaves were severely affected, so that at the time of final sampling, apart from a few older leaves, all tissue was of a uniform pale yellowish green colour. In addition, the stems and midribs of the tomato, and to a lesser extent, the white clover plants, bore a purple pigmentation. The leaf areas and stem and petiole diameters were much reduced, and more particularly in the white clover, the stems and petioles had become stiff and woody. These symptoms agreed with those reported by Nightingale, Schermerhorn, and Robbins (1932) and Eaton (1935, 1941, 1942) in tomato, soybean, sunflower, and mustard.

(c) *Analytical Procedure*

Analyses of normal and deficient plants were made when the deficient plants showed severe deficiency symptoms. White clover grown by successive weekly plantings was sampled 7 weeks after germination. Material from the other species was taken from plants grown at a single sowing, at intervals of 7 days. Fresh leaves, and in some instances, whole tops (1.5 g) were ground and extracted with 75 per cent. ethanol. The extract (20–25 ml) was evaporated to dryness under reduced pressure at about 35°C and stored over P_2O_5 .

In preparing two-dimensional chromatograms of the free amino acids, the extract was dissolved in 200 μl water and 10 μl of this solution applied to the papers. The chromatographic development was effected after the method of Dent, Stepka, and Steward (1947) with water-saturated phenol as first solvent and 2,4/2,5 lutidine-water (100:80) as second solvent. The separation in lutidine was carried out at 10°C. The amino acids were detected on the paper by spraying with 0.1 per cent. ninhydrin in ethanol and heating at 90°C for 7–10 min. Quantitative determinations were not attempted, but an indication of the relative amounts of the amino acids on the chromatograms was obtained from the sizes and colour intensities of the spots which were graded into nine intensity groups. In the case of *D. uncinatum* a more precise estimate was made of the proportionate increase of arginine in the sulphur-deficient plants. This was done by comparing the spot areas and colour intensities of the

ninhydrin compound on a number of chromatograms prepared from a series of dilutions of the extract of the deficient plants, with that of the standard amount of the extract of the normal plants. The amino acids were identified by chromatographic comparison with known substances. The identity of arginine was confirmed by means of the Sakaguchi reaction (Consden, Gordon, and Martin 1946). Citrulline was first detected by the ninhydrin reaction as a spot on the chromatograms situated between glutamine and γ -aminobutyric acid, though it somewhat overlapped and had travelled slightly further than glutamine in lutidine. It was distinguished from β -alanine, which also occurs approximately in this position, by spraying with Erlich's reagent (Dent 1948). The identity of the citrulline was confirmed by production of the 2,4-dinitrophenyl derivative, using the procedure first introduced by Sanger (1945), followed by chromatographic comparison with the 2,4-dinitrophenyl derivative of synthetic citrulline.

Sulphur-deficient flax plants (30 g fresh weight) were extracted with 75 per cent. ethanol. Inorganic anions and non-electrolytes were removed by passing the extract through an "Amberlite IR-120" column (38×1.5 cm, containing 54 g moist resin) in the hydrogen form and washing with water. The column was then washed with $N \text{ NH}_4\text{OH}$; this displaces all the amino acids except arginine, which is however eluted under these conditions. The movement of arginine was followed by means of the Sakaguchi reaction. After evaporation to dryness the solid material was dissolved in water and again applied to the regenerated column. The acidic and neutral amino acids were eluted with $N \text{ HCl}$ (250 ml), the column washed with water to remove chloride ions, and the basic amino acids displaced with $0.1N \text{ NH}_4\text{OH}$ (100 ml). The eluate was collected in 5-ml fractions. The fractions giving positive Erlich's reaction were bulked, evaporated to dryness under reduced pressure, and dissolved in water. The solution was then passed through an "Amberlite IR-410" column, (9×0.9 cm, 5 g moist resin), in the hydroxyl form and the column washed with water. A trace of arginine which remained was, by this means, separated from the other amino acids, since it was not taken up. Basic substances absorbed by the column were displaced with $0.1N \text{ HCl}$, the hydrochloric acid removed from the eluate, and the amino acids dissolved in 4 ml water.

By adding sodium carbonate (40 mg) and 1,2,4-fluorodinitrobenzene (39 mg) in ethanol (1 ml) to 1 ml of this solution, dinitrophenyl derivatives of the amino acids were prepared. The mixture was shaken for 2 hr at 40°C , the ethanol removed, and the aqueous solution diluted to 5 ml and extracted with ether (5×2 ml) to remove excess of the fluorodinitrobenzene. After acidification with $6N \text{ HCl}$ to pH 2 the aqueous solution was extracted with (5×5 ml) 5 per cent. butanol in benzene (v/v). The 2,4-dinitrophenyl derivatives not extracted by this solvent were extracted with 10 per cent. $6N \text{ HCl}$ in methyl ethyl ketone (3×2 ml) and the acid removed under reduced pressure. The derivatives from the ketone extraction were then dissolved in 15 per cent. butanol in benzene (v/v). One-dimensional chromatograms of the 2,4-dinitrophenyl derivatives of both the butanol-benzene and the methyl ethyl ketone extracts were prepared on Whatman No. 1 filter paper, using the two separate solvent systems toluene-chloroethanol-pyridine- $0.8N$ ammonia ($5 : 3 : 1.5 : 3$) and $1.5M$ aqueous phosphate buffer ($M \text{ NaH}_2\text{PO}_4 + 0.5M \text{ Na}_2\text{HPO}_4$) (Levy 1954). The

presence of citrulline was confirmed by chromatographic comparison of the 2,4-dinitrophenyl derivative of synthetic citrulline in both solvent systems with the derivative from the plant extract. The 2,4-dinitrophenyl derivative of citrulline occurred chiefly in the methyl ethyl ketone extract. The R_F values, while somewhat variable, were of the order of 0.1 in toluene-chloroethanol-pyridine-ammonia and 0.4 in aqueous phosphate buffer. That lysine also occurred was indicated by the presence of a weak spot, R_F approximately 0.8 in toluene-chloroethanol-pyridine-ammonia, and 0.05 in the phosphate buffer (Levy 1954).

III. RESULTS

Figure 1 shows the effect of sulphur starvation on the free amino acids of the five species examined. The data are arranged in blocks according to treatment. Each

	D. UNCINATUM						WHITE CLOVER				TOMATO						BARLEY						FLAX					
	NORMAL			S-DEF.			NORMAL		S-DEF.		NORMAL			S-DEF.			NORMAL			S-DEF.			NORMAL			S-DEF.		
	5*	6	7	5	6	7	7	7	7	7	5	6	7	5	6	7	5	6	7	5	6	7	6	7	8	6	7	8
	5	6	7	5	6	7	7	7	7	7	5	6	7	5	6	7	5	6	7	5	6	7	6	7	8	6	7	8
ASPARTIC ACID	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
GLUTAMIC ACID	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
ASPARAGINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
GLUTAMINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
SERINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
GLYCINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
THREONINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
α -ALANINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
γ -AMINOBUTYRIC ACID	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
ARGININE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
PROLINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
VALINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
LEUCINE (S)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
TYROSINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
PHENYLALANINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
CITRULLINE	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22

* AGE IN WEEKS WHEN SAMPLED

Fig. 1.—Effect of sulphur starvation on the free amino acids of five plant species.

column within a block shows the results from a single plant extract; in most instances of course, it represents only a typical result from more than one extract examined. The intensities of the various spots on the chromatograms due to amino acids (derived always from a constant fresh weight of plant material), are represented by the numbers of dots (3–27) entered in the diagram. A blank space indicates that no ninhydrin spot could be detected.

It should be remembered that in the diagram the numbers of dots used to indicate the intensity groups form a linear series, whereas the intensity groups themselves, since they depend on visual judgment, presumably represent an exponential series much more nearly than a linear series in terms of colour on the chromatograms, and therefore of quantity of amino acid present; i.e. as a visual impression the diagram underrates the colour differences to be seen on the chromatograms between the higher intensity groups relative to those between the low intensity groups. Bearing this in mind, the method used here to represent roughly the amounts of the substances gives a reasonably accurate picture of their actual variations in so

far as any single amino acid is concerned; but owing to the individual behaviours of the different amino acids in the ninhydrin reaction, the assessment of the colours does not, of course, always properly reflect the relative amounts of two different substances.

Earlier workers have found that sulphur deficiency in plants results in an increase in the free amino acids and amides. This effect can clearly be seen from the figure. However, not all the amino acids are increased as a result of reduced sulphur supply. The high arginine content in sulphur-deficient *D. uncinatum* is particularly striking. The amount of this substance in the deficient plants was estimated to be 150 times greater than that found in the normal plants. Glycine, serine, and asparagine were also significantly increased. On the other hand, the glutamic acid content was much reduced. Glutamine, which occurred in trace amounts only in the normal plants, was not appreciably changed by the treatment. The effect of sulphur deficiency on white clover compared very closely with *D. uncinatum*, though the changes in the amino acid contents were less pronounced. The arginine accumulation in the leaves was relatively slight; however, it is of interest that this substance occurred in quite high concentration in the roots of these plants.

Of the three remaining species, perhaps the outstanding effect of the treatment was the extremely large accumulation of glutamine in the sulphur-deficient tomato plants. The increase of this substance in these plants reached almost the proportions of arginine accumulation in *D. uncinatum*. In these plants too there was a marked reduction in the amount of glutamic acid and increases of arginine, glycine, serine, and asparagine occurred. Less pronounced effects were seen in the deficient barley plants. Arginine was again increased, though in minor amount, and there were increases of glutamine, asparagine, glycine and serine, while the amount of glutamic acid in these plants was appreciably less than that in the normal plants. While sulphur deficiency in flax resulted in a high arginine accumulation, and some increase in glycine, the remaining amino acids were much less affected by the treatment in this than the other species. However, citrulline, which has not been detected in normal flax, was shown to be present in the deficient plants.

Though the amino acid changes resulting from sulphur starvation in all five species examined had many features in common there were also some distinctive effects for, while amide accumulation occurred in each instance, this accumulation was not attributable to a common amide. A marked increase in asparagine was found in the deficient leguminous species, glutamine in tomato, and to a minor extent in flax, while an equal amount of both amides appeared in barley. It is of interest that the amide present in greatest amount in the deficient plants was that which predominated in the normal plants.

IV. DISCUSSION

This paper establishes that sulphur-deficient white clover, *D. uncinatum*, tomato, and flax plants have a high arginine content. This finding is in conformity with the work of Mertz, Singleton, and Garey (1952) who showed that this amino acid occurred in increased amount in sulphur-starved alfalfa. Arginine accumulation, which varies in degree between species (only a small increase was found in barley), may well be associated with sulphur deficiency in many plants.

Deficiencies of some other essential elements have also been associated with arginine accumulation. Richards and Berner (1954) have shown that this amino acid occurs in increased amount in potassium-starved barley. A similar effect was reported by Holley and Cain (1955) who found a higher concentration of this substance in the leaves of blueberry, apple, and magnolia plants affected by an iron-deficiency type chlorosis than in the leaves of normal plants. Deficiencies of potassium, iron, and sulphur have each been associated with a decrease in protein and an increase in the free amino acids of plants. Hewitt, Jones, and Williams (1949) reported that manganese deficiency in cauliflower resulted in increased arginine, though it is not known if this deficiency is concerned with protein metabolism. The accumulation of arginine in common with these deficiencies is of interest alike to those concerned with pathways in amino acid metabolism, and with the functions of the elements within the plant.

Mertz, Singleton, and Garey (1952) suggested that the accumulated arginine in sulphur-deficient alfalfa may be concerned with nitrogen storage. In this connection, in a small series of experiments, the author has found a higher content of free arginine in the rhizomes of bracken than in the fronds, although whether this was related to storage is not known. However, free arginine has rarely been found in high concentration in normal plants, and its association with mineral deficiencies suggests that its appearance in supranormal amounts results directly from a disruption of nitrogen metabolism. Either the increased amino acid is derived from protein breakdown, or a block in amino acid metabolism has occurred. The latter could be associated either with the failure of some essential function of an element, or, in the case of sulphur, with the lack of a sulphur containing metabolite. Further evidence is needed before this matter can be clarified.

Referring to the origin of free arginine in normal plants, apart from that arising through proteolysis, work in progress shows that both white clover and barley accumulate this amino acid when given citrulline through the roots or stems. Experiments with L-citrulline- ^{14}C should establish whether arginine is in fact derived from the citrulline supplied. Significantly, sulphur-deficient flax contains citrulline in addition to increased arginine.

In connection with amide accumulation, it is of interest that while the free amide in the deficient plants was relatively high, the asparagine and glutamine contents of different species varied considerably. Asparagine predominated in sulphur-deficient white clover and *D. uncinatum*, glutamine in tomato and flax, while barley contained about equal amounts of both substances. Vickery, Pucher, and Deuber (1942), working with normal plants, found asparagine to be the prominent amide of lupine and soybean seedlings, while glutamine was prominent in squash (*Cucurbita pepo* L.) (Vickery and Pucher 1943). Noteworthy is the fact that in the present work the predominant amide in the normal plant was also the amide which accumulated in the deficient plant, that is asparagine in leguminous species and glutamine in tomato. Further, the metabolism of glutamic and aspartic acids may be mentioned. Glutamine is readily formed from glutamic acid and ammonia in many plants (Webster 1953) and such a conversion may well explain the high glutamine content of sulphur-deficient tomato leaves, where glutamic acid was

greatly depleted. Asparagine is similarly formed from aspartic acid and ammonia in some plants (Webster and Varner 1955), and this may be the pathway of asparagine metabolism in the sulphur-deficient leguminous species. However, further facts are needed to explain the differences in amide metabolism noted between the legume *D. uncinatum*, where no glutamine was accumulated in the deficient plants despite the fact that a pronounced reduction of glutamic acid had occurred, and that of tomato, where a very significant accumulation was associated with a comparable reduction of glutamic acid.

V. ACKNOWLEDGMENTS

The author wishes to thank Dr. J. Griffiths Davies for his encouragement during these investigations, and members of the Laboratory for their friendly cooperation. In particular thanks are due to Mr. C. S. Andrew who provided some of the plant material.

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NUTRIENT INTERACTIONS AND DEFICIENCY DIAGNOSIS IN THE LETTUCE

II. EFFECTS OF NUTRITION ON WATER CONTENT

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[Manuscript received August 8, 1956]

Summary

In a sand-culture experiment with lettuces, the water content (as per cent. of dry matter) of the aerial portions was studied in plants subjected to five levels of nitrogen, phosphorus, and potassium supply in all combinations.

The higher levels of nitrogen supply were found to decrease the water content, while increases in phosphorus supply had the reverse effect. Plants deficient in potassium had a higher water content than those receiving an adequate amount, unless phosphorus was also deficient.

I. INTRODUCTION

The present series of papers describes an experiment designed primarily to study the relationship between the content of nitrogen, phosphorus, and potassium in young lettuce plants, on the one hand, and their subsequent response to nutritional supplements of these elements on the other. A wide range of nutrient combinations in sand culture was used to obtain plants differing greatly in their nutritional status, and hence in the responses which might be expected. This wide range of treatments (five levels of nitrogen, phosphorus, and potassium in all combinations, giving 125 treatments in all) makes the results of considerable interest apart from the main purpose of the experiment; such extensive sets of data on the effects of nutrient interactions are uncommon. Accordingly, the first paper of this series (Goodall, Grant Lipp, and Slater 1955) was devoted simply to a consideration of the dry weight results, and the present paper deals similarly with water content; the nutrient content of the plants, and its relationship to the responses shown, will be considered in subsequent papers.

We will proceed directly to discussion of the water-content data, since the design of the experiment, and the methods used, were fully described in the first paper. As previously, the data are not presented in full here, but the results have been tabulated *in extenso* in the original theses (Grant Lipp 1952; Slater 1952) on which these papers are based.

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TABLE 1
WATER CONTENT OF TOPS (PER CENT. OF DRY MATTER): ANALYSES OF VARIANCE

Days from Sowing	Source of Variation	Degrees of Freedom	Mean Square	<i>F</i>
22	N	2	90,626	5.78*
	P	2	936,562	59.76***
	K	2	82,337	5.25*
	N × P	4	6,869	—
	N × K	4	11,889	—
	P × K	4	8,390	—
	Error	8	15,673	
29	N	4	109,920	1.32
	P	4	4,568,593	54.94***
	K	4	618,746	7.44***
	N × P	16	157,002	1.89*
	N × K	16	38,408	—
	P × K	16	88,532	1.06
	Error	64	83,149	
37	N	4	166,334	3.15*
	P	4	2,984,193	56.49***
	K	4	305,682	5.79***
	N × P	16	87,950	1.67
	N × K	16	55,146	1.04
	P × K	16	98,142	1.86*
	Error	64	52,826	
44	N	4	143,158	5.51**
	P	4	1,738,162	66.96***
	K	4	217,120	8.36***
	N × P	16	89,384	3.44***
	N × K	16	25,459	—
	P × K	16	74,052	2.85**
	Error	64	25,960	
98†	N	4	655,463	3.89*
	P	2	753,190	4.47*
	K	4	155,890	—
	N × P	8	280,678	1.67
	N × K	16	381,096	2.26*
	P × K	8	70,358	—
	Error	27	168,406	

* $P = 0.01-0.05$.

** $P = 0.001-0.01$.

*** $P < 0.001$.

† Plants without sub-treatment only.

II. RESULTS

The water-content results are expressed as percentages of dry matter. Those reported here all relate to the aerial portion (leaves and stem) considered as a whole. Results for older and younger leaves separately were available for many plants, but these data were not numerous enough to justify discussion here. Fresh weights had not been determined for the seedlings harvested at 11 days. At 98 days, many of the plants receiving the low-phosphorus (P_1 and P_2) treatments had died, and only for the higher phosphorus levels were results sufficiently complete to justify study; since the means for 98 days are thus not fully comparable with those for earlier harvests, they are omitted from the diagrams. At 22 days, water contents were determined only for a selection of treatments (the combinations of levels 1, 3, and 5 of each nutrient). The analyses of variance for each harvest are presented in Table 1, and the means for those treatment effects and interactions which proved to be significant are shown in Table 2.

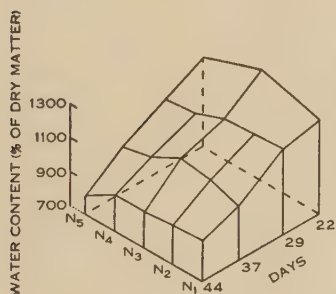


Fig. 1

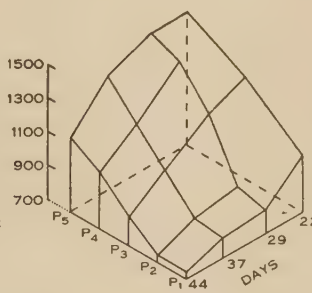


Fig. 2

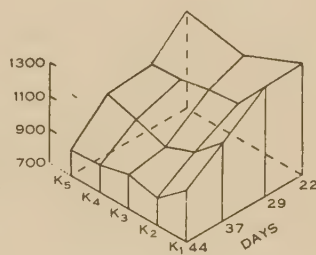


Fig. 3

Figs. 1-3.—Effect of nitrogen (Fig. 1), phosphorus (Fig. 2), and potassium (Fig. 3) supply on water content (per cent. of dry matter) at different stages of development.

(a) *Nitrogen Effects*

In all harvests except that at 29 days, the effect of nitrogen supply on water content was significant (Fig. 1). The highest nitrogen levels (N_4 and N_5) always resulted in a lower water content, but differences among the plants receiving the lower levels of nitrogen supply (N_1 – N_3) were inconsistent.

(b) *Phosphorus Effects*

Phosphorus had the most marked effect of all three nutrients on water content, as on dry weight (Fig. 2). This was significant at all stages, and consisted in a progressive increase in water content with increasing phosphorus supply. With P_5 , the mean water content was, after 22 days, more than half as much again as with P_1 .

(c) *Potassium Effects*

The effect of potassium (Fig. 3) was significant on each occasion except the last. It will be recalled that the K_1 pots received no potassium, and it was found that these plants had a consistently higher water content than the others. Increase in potassium supply above K_2 had little effect on water content.

TABLE 2

MEAN EFFECTS OF NUTRIENT TREATMENTS ON WATER CONTENT OF TOPS (PER CENT. OF DRY MATTER)

22 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

N ₁	1264	P ₁	1045	K ₁	1358
N ₃	1364	P ₃	1312	K ₃	1224
N ₅	1227	P ₅	1499	K ₅	1274

29 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

N ₁	1212	P ₁	832	K ₁	1357
N ₂	1202	P ₂	875	K ₂	1153
N ₃	1180	P ₃	1211	K ₃	1135
N ₄	1122	P ₄	1422	K ₄	1100
N ₅	1109	P ₅	1485	K ₅	1080

Interactions between Initial Treatments

	N ₁	N ₂	N ₃	N ₄	N ₅
P ₁	880	786	931	801	762
P ₂	1053	863	900	771	790
P ₃	1355	1225	1289	993	1196
P ₄	1489	1414	1388	1399	1422
P ₅	1286	1722	1392	1649	1375

37 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

N ₁	1074	P ₁	817	K ₁	1184
N ₂	1084	P ₂	834	K ₂	1013
N ₃	1115	P ₃	1022	K ₃	996
N ₄	1016	P ₄	1200	K ₄	1005
N ₅	971	P ₅	1388	K ₅	1063

Interactions between Initial Treatments

	P ₁	P ₂	P ₃	P ₄	P ₅
K ₁	752	890	1240	1481	1557
K ₂	701	786	1051	1170	1357
K ₃	877	783	961	1108	1250
K ₄	863	800	954	1104	1303
K ₅	891	910	904	1137	1475

TABLE 2 (Continued)

44 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

N ₁	939	P ₁	723	K ₁	1012
N ₂	933	P ₂	736	K ₂	867
N ₃	914	P ₃	864	K ₃	909
N ₄	912	P ₄	1037	K ₄	868
N ₅	808	P ₅	1146	K ₅	850

Interactions between Initial Treatments

	P ₁	P ₂	P ₃	P ₄	P ₅
N ₁	751	750	1063	1159	971
N ₂	682	803	898	1108	1172
N ₃	719	757	826	1051	1219
N ₄	734	690	840	1041	1256
N ₅	727	680	693	825	1114
	P ₁	P ₂	P ₃	P ₄	P ₅
K ₁	648	744	1053	1222	1395
K ₂	774	703	830	999	1027
K ₃	728	738	894	1029	1156
K ₄	723	791	801	984	1042
K ₅	742	704	741	950	1112

98 Days from Sowing†

Mean Effects at Different Levels of Initial Treatments

N ₁	1059			K ₁	1296
N ₂	1587			K ₂	1304
N ₃	1220	P ₃	1117	K ₃	1103
N ₄	1103	P ₄	1146	K ₄	1136
N ₅	1187	P ₅	1431	K ₅	1315

Interactions between Initial Treatments

	K ₁	K ₂	K ₃	K ₄	K ₅
N ₁	1265	927	964	1098	1040
N ₂	1411	1498	1226	1545	2254
N ₃	998	1325	1479	1116	1188
N ₄	1229	1417	970	967	932
N ₅	1568	1366	887	955	1161

† Plants without sub-treatment only.

(d) Nutrient Interactions

The interaction between nitrogen and phosphorus (Fig. 4) is significant at 29 days and highly significant at 44 days. At the latter harvest, the change from an optimum phosphorus level of P_4 with low nitrogen to P_5 at the higher levels of nitrogen supply may be clearly seen in the diagram; and there seems to be a tendency in the same direction at earlier harvests.

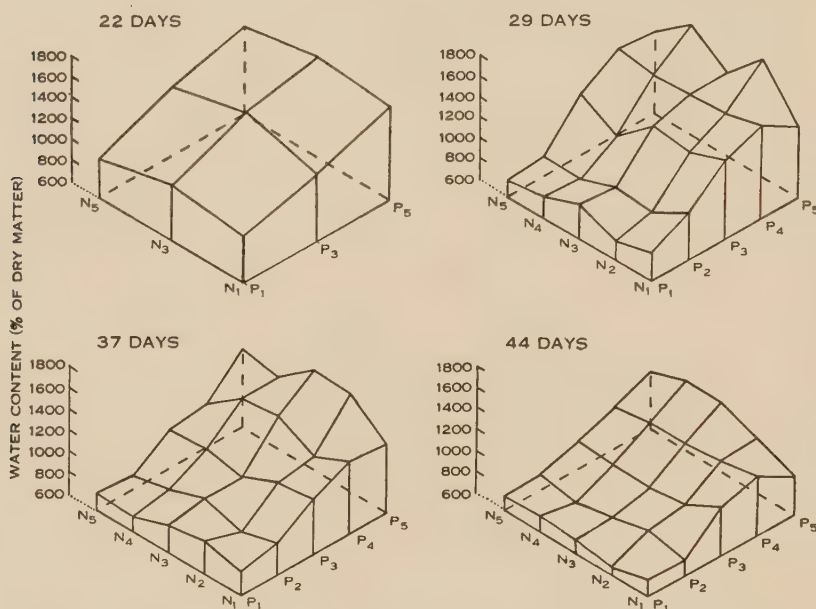


Fig. 4.—Interaction effects of nitrogen and phosphorus supply on water content (per cent. of dry matter).

The interaction between phosphorus and potassium (Fig. 5) was also significant on two occasions (at 37 and 44 days), and the same trend was already evident at the previous harvest. This interaction is easier to interpret than that between nitrogen and phosphorus; the effect of potassium supply in reducing water content was apparent only at higher phosphorus levels, and non-existent in plants receiving the lowest phosphorus supply (P_1).

The interaction between nitrogen and potassium was significant only at 98 days, and then was obscure, seeming to depend on a single aberrant value in N_2K_5 .

(e) Sub-treatments

In no case were significant changes in water content observed as a result of the additional supplies of nutrients added at 46 days; but it should be remembered that the plants most seriously deficient in phosphorus (P_1 and P_2) were perforce omitted from the analysis of the final results.

III. DISCUSSION

The effects of nutrition on the water content of plants have often been studied, but few investigations have been so designed as to enable the effects of nutrient interactions to be determined. The most noteworthy relevant set of observations on the lettuce is that of Woodman (1939*a*, 1939*b*, 1940*a*, 1940*b*); but, again, interactions were not studied.

In the present results, the effect of nitrogen is rather surprising. Additional supplies of nitrogen are usually found to increase the water content of plant tissue (see, for instance, Gregory and Richards 1929), while reducing the thickness of cell walls and the proportion of mechanical tissue. Here, on the contrary, high levels of

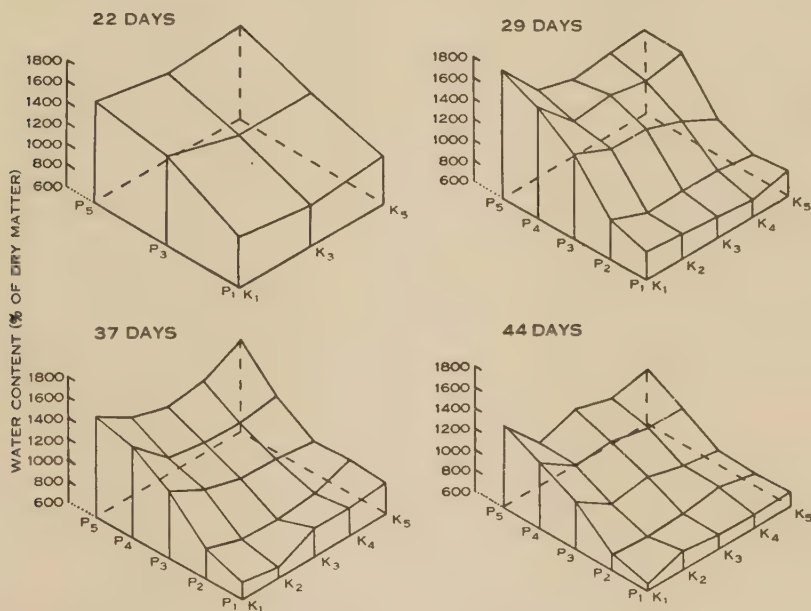


Fig. 5.—Interaction effects of phosphorus and potassium supply on water content (per cent. of dry matter).

nitrogen reduced the water content—in direct contradiction of the results obtained by Woodman (1939*b*, 1940*a*) with the same species. In comparing the present results with those of Woodman, however, it should be borne in mind that Woodman's related to plants adequately supplied with other nutrients, whereas the majority of our own plants were more or less deficient in phosphorus. Further, the highest nitrogen supply, N_5 , seems to have been definitely supra-optimal. If only plants with suboptimal nitrogen and adequate phosphorus are considered, the general trend is in agreement with that reported by Woodman and others.

The effect of phosphorus—in the reverse direction to that of nitrogen—is also somewhat surprising since, in these same plants, high levels of phosphorus supply led to increases in nitrogen content (Slater and Goodall 1957). One must conclude that the effect of phosphorus on water content is a specific one, independent of its

effect on nitrogen uptake. The increase in water content with phosphorus supply is, however, in accord with previous data for lettuce (Woodman 1939*a*, 1940*a*), and with the results for barley obtained by Gregory and Richards (1929), Richards and Shih (1940*a*), and Richards (1944).

The effects of potassium on water content have been the subject of some controversy in the literature. Janssen and Bartholomew (1929, 1930) and Wallace (1931), for instance, found potassium to increase water content; Woodman (1940*a*) with lettuce and James (1930) with potato concluded it had little effect; while Gregory and Richards (1929, see also Richards 1932) found a marked decrease from a high water content under potassium deficiency—in agreement with the present lettuce data. Later studies (Richards and Shih 1940*a*, 1940*b*; Richards 1944) led to the conclusion that the discrepancies were due to other ions in the culture solution, and that the effects of potassium deficiency could in many cases be ascribed to the high sodium content of these plants. This possibility could not be tested for the experiment now under discussion, for no sodium analyses were performed; but no additional sodium had been supplied to the potassium-deficient plants.

The interaction effects between potassium and phosphorus may be compared with those found by Richards and Shih (1940*a*) for barley. They concluded that “*the decrease in succulence associated with reduced phosphorus supply is negligible at high potassium levels, but increases markedly as potassium supply is lowered.*” In the present data for lettuce, the effect of phosphorus was marked at all potassium levels, but was greater under potassium deficiency. To put it the other way round, the potassium effect was evident only when phosphorus was not severely deficient.

IV. ACKNOWLEDGMENTS

We have pleasure in expressing our thanks to our Melbourne colleagues mentioned in the first part of this series, and particularly to Professor J. S. Turner, in whose department the work was conducted, for his constant encouragement. Miss Julie Bromley, University of Reading, was responsible for the diagrams. The work was financed from the Research Grant of the University of Melbourne.

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CHLORINE AND BROMINE IN THE NUTRITION OF HIGHER PLANTS

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[Manuscript received September 4, 1956]

Summary

Plants were cultured in purified inorganic salt solutions and with filtered glass-house air to reduce contamination by chlorine. Leaf and top yields of tomato and clover respectively were reduced by over 80 per cent. when insufficient chlorine was supplied.

Supplying bromine in place of chlorine gave yields to 75–95 per cent. of the yields with chlorine. However, plants receiving bromine always extracted more chlorine from their environment. In some instances, five times as much chlorine appeared in plants treated with bromine as in “no bromine” controls. In all cases the yields with bromine applications were less than those with applied chlorine. Bromine does not appear to completely substitute for chlorine in growth and development of plants, as evidenced by weight and wilt differences. Part of the apparent effect of bromine was due to chlorine arising from extraneous contamination, e.g. the atmosphere. When plants were given ample chlorine, no yield differences were obtained by additions of bromine.

Without added halide, foliar concentrations of chlorine were as low as 1.0 $\mu\text{g-atom/g}$ dry weight for tomatoes, and 0.8 $\mu\text{g-atom/g}$ for clover. With ample bromine supplied, these values fell only to 0.7 and 0.6 respectively. Large decreases in tomato yields were found when leaf chlorine concentrations fell below 2.0 $\mu\text{g-atoms/g}$ dry weight.

The addition of bromine to roots of intact plants in a “minus chloride” culture solution caused loss of chlorine from the roots. Similarly, with decapitated plants, chlorine was found to be displaced, moving upwards in the exuding sap. It is inferred that the effect of bromine supplied to chlorine-deficient plants is due to displacement of the chlorine differentially from non-effective places to more essential loci.

I. INTRODUCTION

The recent demonstration (Broyer *et al.* 1954) that chlorine is necessary for growth of higher plants has opened up a number of questions with respect to this element in nature. From one point of view, since chlorine is a plant micronutrient, it becomes of interest to learn more of its occurrence and availability to plants in their natural settings. There are also questions of its quantitative requirements for different plant species and of the functions of chlorine in plant metabolism including possible interrelationships with other plant nutrients.

A finding of immediate interest is the degree to which bromine can substitute in plant functions normally dependent upon chlorine. Perhaps only potassium, among the presently recognized essential elements for higher plants, can go as far toward partially sharing functions with another element—as, for example, with sodium.

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Tomato plants cultured in rigorously purified salt solutions provided the first clear-cut demonstration of the requirement for chlorine (Broyer *et al.* 1954) and similar cultural techniques were successfully extended to a number of other species (Johnson *et al.* 1957). Chlorine deficiency is characterized by leaflet wilting, followed progressively by chlorosis, bronzing, and finally, necrosis. These symptoms could be prevented by providing the culture solutions with small amounts of chloride ion. It was also noted that additions of bromide delayed the onset of chlorine deficiency, but the efficiency of bromine was less. Consequently, it was tentatively concluded that bromine exhibited a "sparing effect" for at least some functions normally met by chlorine. The effect of bromine in alleviating chlorine deficiency has been confirmed by Ulrich and Ohki (1956).

Thus, when the studies to be described here were commenced, it appeared evident that chlorine activates some essential function in plants and that bromine exercised a sparing effect for chlorine. It was not clear how far bromine could serve as a substitute for chlorine. Also, it remained to be shown whether or not bromine played some necessary role distinct from that of chlorine. In the six experiments to be described, both highly purified salt solutions and filtered air were used during plant cultivation, with the result that concentrations of chlorine in plant tissues were reduced well below those reported earlier. New evidence on the relative effectiveness of chlorine and bromine is presented.

II. MATERIALS AND METHODS

(a) Culture Technique

The method used in these studies was to culture four tomato plants (*Lycopersicon esculentum* Mill. var. Marglobe) or six clover plants (*Trifolium subterraneum* L.) per 4 l. of inorganic salt solution. For further details of containers etc. see Broyer *et al.* (1954). Seeds were germinated on cheesecloth over dilute culture solution "free" from chlorine and bromine, then transplanted to 4-l. beakers when the seedlings had roots 2-3 in. long. The culture solutions were aerated continuously during plant growth.

All experiments were carried out in a glass-house fitted with charcoal and granular limestone filters through which much of the air was drawn into the glass-house. At harvest, rubber gloves were used in the separation of the plant material. Roots were rinsed in distilled water and spun in a centrifuge. All material was dried for 48 hr in a forced draught oven at 90°C. Dry weights were taken and the samples ground to pass a 40-mesh sieve prior to analysis.

In these experiments, two types of contamination are recognized. The first, *intrinsic* to the plant-culture system, includes known contamination from seed, in the repurified salts and water, and from aeration. The second, *extrinsic* to the system, includes that from airborne or other sources unknown or uncontrolled.

(b) Culture Solutions

The culture solutions were all compounded on the chemical equivalency basis as proposed by Johnson *et al.* (1957). The M6 fraction contained the six macro-nutrient ions in m-moles/l as follows: Ca^{++} , 4; Mg^{++} , 1; K^+ , 6; NH_4^+ , 2; $\text{PO}_4^{=}$, 2;

$\text{SO}_4^{=}$, 1; and NO_3^- , 14. For culturing clover, the ammonium and phosphate ions were each reduced to a concentration of 1 m-mole/l. The (m7—Cl) micronutrient fraction (Johnson *et al.* (1957), Table 1), providing six micronutrient elements, was added in the following microgram-atoms/l quantities: B, 25; Mn, 5; Fe, 4; Zn, 2; Cu, 0.5; and Mo, 0.1. The iron was added as FeSO_4 at 8 μg -atoms per culture each week. Only analytical reagent grade salts, which had been purified by recrystallizing twice, were used in preparation of the cultures. Solutions so prepared contained less than 2 μg -atoms Cl per culture. During growth, the plants obtained a continual small supply of chlorine apparently from contamination from the atmosphere, and from the glass-redistilled water. This contamination increased with plant size. Contamination with bromine during growth appeared to be negligible. Where chlorine and bromine were added to the solutions, twice recrystallized reagent grade potassium salts were used. Maximum chlorine in the recrystallized potassium bromide was less than 1 μg -atom per culture in the highest bromine level supplied (2000 μg -atoms per culture).

The plants cultured without added chlorine or bromine are referred to as M6+(m7—Cl) controls and received only the "chlorine" intrinsically derived from the purified solutions and extrinsically derived from atmosphere sources. In 6-week-old plants this contamination chlorine varied from 10 to 30 μg -atoms per culture.

(c) Analytical Methods

Chlorine and bromine in the plant material were measured by modifications of the Conway (1947) microdiffusion technique, after incomplete ashing at 550°C in the presence of potassium carbonate.

When "tracer" amounts of radiochlorine were used as treatments, they were applied as ^{36}Cl . This was measured by use of a thin end-window Geiger tube (1.7 mg/cm²).

III. OBSERVATIONS AND RESULTS

(a) Comparative Plant Growth Effects of Chlorine and Bromine

In experiment 1, tomato plants were cultured over a wide range of halide supply levels—one series having 0, 2, 8, 30, 100, 300, and 1000 μg -atoms Cl applied per culture, and the other series 0, 10, 30, 100, 1000, and 2000 μg -atoms Br applied per culture. Three replicates of each treatment level were prepared. Acute chlorine-deficiency symptoms as described by Broyer *et al.* (1954) were observed in the lower halide cultures. Symptoms of leaf wilting and bronzing did not appear however at the higher levels of chlorine, i.e. 30 μg -atoms per culture and above. On the bromine treatments of 100 μg -atoms per culture or more, no bronzing occurred, although occasionally, under conditions of enhanced water stress, wilting occurred even at the highest level of bromine. After 6 weeks growth the trial was terminated and the plant material separated into roots, stems, and leaves.

Data showing yields of roots, stems, and leaves at the various levels of each applied halide are presented in Figure 1. Marked effects of treatment were obtained in all plant parts, although the leaf weights were affected more than the stems from

applied halide, while the root weights increased least of all. Both chlorine and bromine application gave effectively the same general yield pattern. However, the

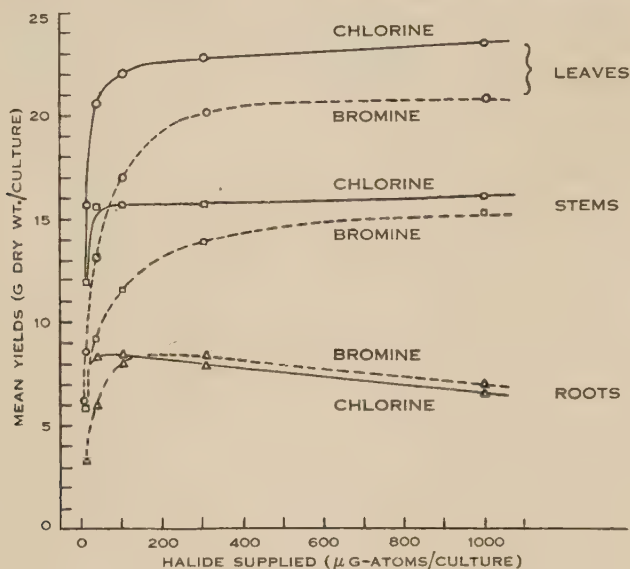


Fig. 1.—Yields of tomato plants when chlorine or bromine is added to the culture solutions in increasing amounts.

effect of added chlorine on the leaves and stems was greater than that of bromine and was obtained at a much lower level of application. Also, the maximum leaf

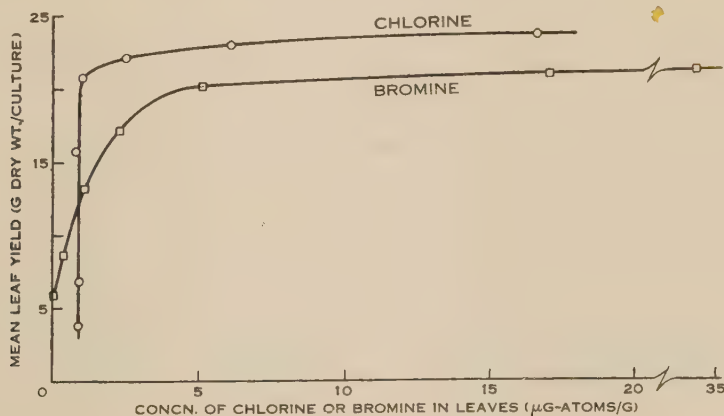


Fig. 2.—Relationship in tomato plants between concentration of chlorine or bromine present in the leaves (dry basis) and the mean leaf yield per culture.

yields with adequate chlorine were significantly greater than those obtained with ample bromine.

The concentrations of halide in the leaves, together with the related yields from experiment 1, are shown in Figure 2. Leaf yields increased rapidly up to bromine concentrations of $5 \mu\text{g-atoms/g}$ tissue. Further increases of bromine to $34 \mu\text{g-atoms/g}$ tissue showed no toxic effect. The growth effects at deficiency levels of chlorine and bromine are shown in more detail in Figure 3. Yields were not markedly affected

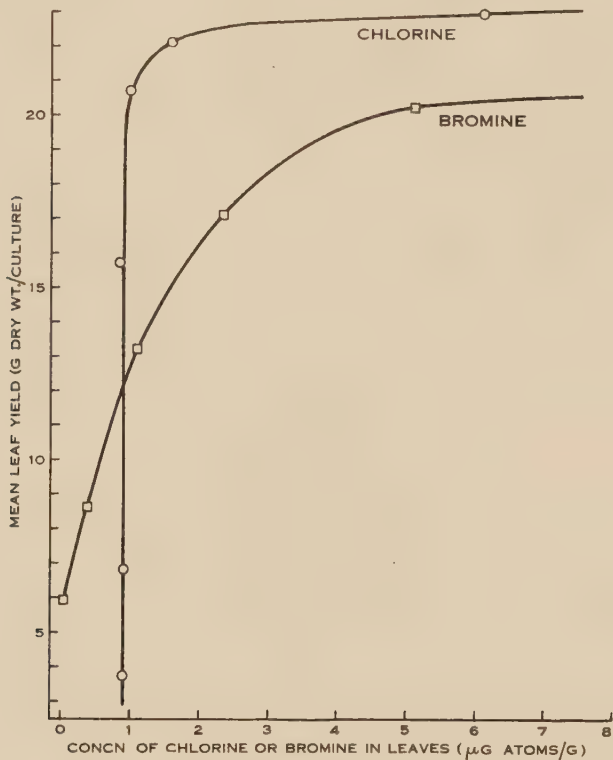


Fig. 3.—Enlarged view of relationship between concentration of chlorine or bromine in leaves and leaf yield at chlorine-deficiency levels (see Fig. 2).

until the leaf-chlorine level became less than $2 \mu\text{g-atoms/g}$ tissue. Then, yields decreased sharply to a minimum at concentrations of $0.9 \mu\text{g-atom/g}$. Further reductions in chlorine supply merely decreased leaf yield without decreasing leaf chlorine concentrations. In the absence of applied bromine, it has not been possible to obtain tomato plants containing chlorine concentrations lower than this value in leaves.

In contrast to the minimal critical threshold of chlorine in the chlorine-yield curves, withholding bromine from plants reduced their bromine concentration to the limit of chemical detection ($0.1 \mu\text{g-atom/g}$ dry weight). However, the concentration of chlorine in the leaves of plants supplied with bromine did not fall below $0.5 \mu\text{g-atom/g}$. The contribution to growth from this contamination chlorine will be discussed later.

It remained to be shown whether additions of bromine to plants which were supplied adequately with chlorine could give further growth effects. To examine this, in experiment 2, 10 cultures of tomato plants were prepared. To all of these, an ample supply (400 μg -atoms per culture) of chlorine was added. In addition, a high level of bromine (1000 μg -atoms per culture) was added to each of five of the beakers. All the plants made healthy growth without deficiency or toxicity symptoms, and were harvested after 6 weeks. The dry weight yields of the plant parts are shown in Table 1. It may be seen that the plants cultured with bromine in addition to chlorine were equal in yield to those supplied chlorine alone. Thus, in the presence of adequate chlorine, bromine neither increased growth through some beneficial function, nor decreased yields by hindering chlorine activity or by some other form of toxicity.

TABLE 1
FAILURE OF BROMINE TO ENHANCE GROWTH OF TOMATOES GIVEN AMPLE CHLORINE
All values are the means of six replicates

Treatment	Yield—Dry Weight (g)			
	Leaves	Stems	Roots	Total
400 μg -atoms chlorine	8.2	5.9	2.4	16.5
400 μg -atoms chlorine + 1000 μg -atoms bromine	8.1	5.9	2.5	16.5
Least significant difference ($P = 0.01$)	1.0	0.9	0.3	2.0

In experiment 3, the relative effects of bromine and chlorine were examined with a different plant species, viz. subterranean clover. In addition to six control cultures without added chlorine, four replications of each of the following treatments were planted: plus chlorine only at 400 μg -atoms per culture; plus bromine only at 400 μg -atoms per culture; and plus both chlorine and bromine, at 400 μg -atoms each per culture. This trial was commenced on November 11, 1955 at Berkeley. Due to slow plant growth in winter, harvest was delayed until February 6, 1956. Chlorine-deficiency symptoms developed on the control plants after 5 weeks, and became acute before the plants were harvested. Shortly after deficiency symptoms developed on the controls, the plus bromine plants became slightly retarded in growth and showed a tendency to wilt on sunny days. The appearance of the respective treatment effects are shown in Plate 1, Figures 1 and 2. The control plants showed marked stunting of roots as well as reduced top growth. Although somewhat larger, the plus bromine plants also had stubby, short, thick roots. The relative root development in the various treatments is shown better in Plate 1, Figure 2. Despite their poor elongation, however, the dry weights of the plus bromine roots were not significantly less than the root weights from the two treatments given chlorine.

This trial was repeated the following summer under more favourable growth conditions. Planted on May 28, 1956, the plants made more growth in 6 weeks than in the 12 weeks in winter. To ensure ample halide supplies, the levels were increased

TABLE 2
 HALOGEN CONTENTS AND YIELDS OF CLOVER TOPS WITH CHLORINE AND BROMINE ADDED TO THE CULTURE SOLUTION
 All values are the means of four replicates

Treatment	Concentrations		Totals		Total Yield (dry wt.) (g)	"Chlorine Yield" (dry wt.) (g)	"Bromine Yield"* (dry wt.) (g)	"Bromine Yield" (% of total)
	Bromine (μ g-atoms/g)	Chlorine (μ g-atoms/g)	Bromine (μ g-atoms)	Chlorine (μ g-atoms)				
Winter								
Control	<0.1	1.42†	—	21.8†	15.5	Nil	Nil	115
Chlorine added	<0.1	13.4†	—	372†	27.9			
Bromine added	15.4†	1.08†	325	22.7†	21.0	5.0	5.0	24
Chlorine and bromine added	13.1†	13.8†	345	363†	26.5			
Least significant difference at $P = 0.01$	2.7†	0.25† 2.7†	41	8.0† 41†	3.9			
Summer								
Control	<0.1	0.77†	—	2.7†	3.3†	Nil	Nil	
Chlorine added	<0.1	13.6†	—	408†	30.0†			
Bromine added	14.5†	0.54†	389	14.5†	26.8†	16.0	16.0	60
Chlorine and bromine added	12.8†	14.6†	389	444†	30.4†			
Least significant difference at $P = 0.01$	2.0†	0.21† 2.0†	31	1.8† 31†	1.6† 3.1†			

* "Bromine yield" by difference: total yield minus "chlorine yield".

†† In some instances two values are given for least significant differences. The treatments to which these values refer are shown by the daggers.

from 400 to 500 μg -atoms per culture. In general, results were similar to those of the winter trial, but the control cultures showed acute chlorine deficiency after only 10 days, and made little growth thereafter. Yields at harvest, for both trials, are given in Table 2, together with the chlorine and bromine concentrations present in the clover tops from the various treatments.

With subterranean clover, the addition of chlorine or bromine or both gave significant increases in plant yields. As was expected from experiment 2, the chlorine and chlorine plus bromine treatments gave similar yields; however, the addition of bromine alone gave plants having significantly smaller tops than did the two treat-

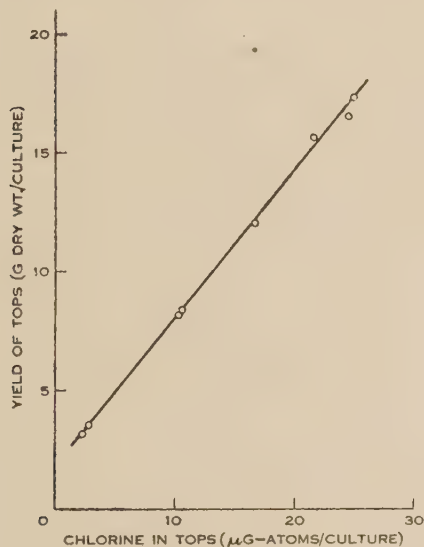


Fig. 4

Fig. 4.—Effect of chlorine content on yield of tops with subterranean clover cultured under very low chlorine-supply conditions.

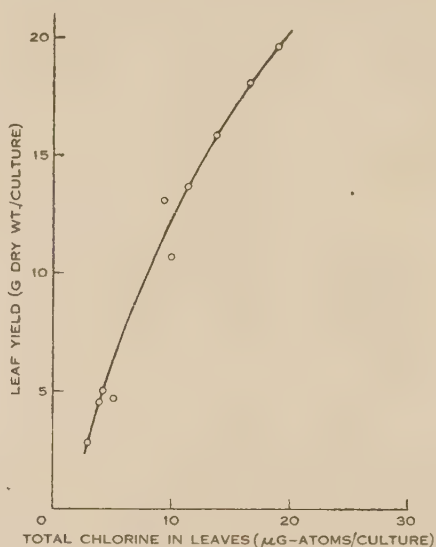


Fig. 5

Fig. 5.—Effect of chlorine content on yield of leaves of tomato plants cultured under very low chlorine-supply conditions.

ments containing added chlorine. Thus, even though bromine caused a great increase in yield in the summer trials, it did not completely substitute for chlorine.

In these trials where chlorine was added, the concentration of chlorine in the plant tops was not decreased by the further addition of bromine. Similarly, the addition of chlorine did not depress bromine uptake.

The control cultures without added chlorine contained 1.42 μg -atoms/g dry weight in winter and 0.77 μg -atom in summer, but the tops of plants supplied bromine still contained appreciable amounts of chlorine, e.g. 1.08 μg -atoms/g in winter, and 0.54 μg -atom in summer. When the total chlorine present in the tops was plotted against top yield for control cultures (without added halide), as in Figure 4, a close linear relationship was found. Each increment in total chlorine from contamination produced an equivalent increase in top weight, so that given a chlorine content it is possible to calculate the approximate "chlorine yield", or dry weight

of tops, which that amount of chlorine would be expected to produce. Assuming this relationship between chlorine and yield holds also for the contamination chlorine in the plants to which only bromine was applied, then in the winter trial it appears that for the plus bromine treatment, 16 of the 21 g top weight could have been produced by the contamination chlorine, leaving only about 24 per cent. of the total yield as the effect of bromine. Similarly, in Table 2, of the 26.8 g yield with applied bromine, only 60 per cent. can be attributed to the bromine present.

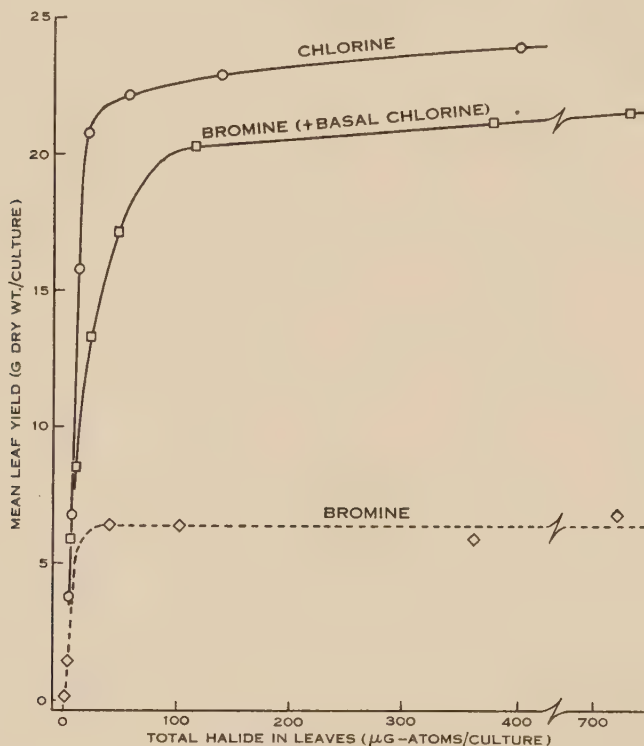


Fig. 6.—Mean leaf yields of tomato plants as modified by halide content. The upper two curves were drawn from experimentally observed values, whilst the lowest was derived from the “bromine (+ basal chlorine)” curve by subtracting the “chlorine yield” as found from Figure 5.

It was decided to use this same method to ascertain how much of the leaf yield from the tomato plants in experiment 1 was caused by the applied bromine. In Figure 5, the leaf yields from the lower chlorine treatments of this experiment are plotted against leaf chlorine content. As with the clover tops, the relationship of leaf weight to chlorine content is very close. After determining the chlorine content of the leaves cultured at different levels of applied bromine, then the yield which this amount of chlorine could have allowed was derived from the graph in Figure 5. The difference between this “chlorine yield” and the total yield was assumed to be the dry weight produced by the bromine present.

The leaf yields plotted against their halide contents are shown in Figure 6. The calculated "bromide yield" curve, shown as a broken line, indicates that the applied bromine did not account for more than about one-third of the total yield—the remainder being produced by the "basal chlorine" received by the plant from the atmosphere and other contamination sources.

(b) *The Differential Displacement of Chlorine by Bromine*

In the trials already described, bromine increased the growth of plants having a limited chlorine supply. It was considered possible that this effect of bromine might

TABLE 3

EFFECT OF BROMINE IN DISPLACING RADIOACTIVE CHLORINE FROM THE ROOTS OF TOMATO PLANTS
Plants were cultured with radiochlorine in the culture solution for 4 weeks, then transferred to "chloride-free" solution and the bromine added. Roots were harvested 2 weeks later

Set	³⁶ Cl Pretreatment (μg-atoms)	Bromine Added (μg-atoms)	Roots	
			Dry Wt.* (g)	Radioactivity* (counts/min)
A	13†	—	3.40	4,350
	13†	400	3.43	3,940
B	80	—	3.83	25,100
	80	400	3.83	23,300
C	154	—	4.17	42,200
	154	400	4.03	36,400
D	154	—	3.80	42,200
	154	400	3.77	33,400

* All values are means of three replicates.

† Chloride level so low that plants developed symptoms of chlorine deficiency.

result from the displacement of chlorine from non-active sites thus making the chlorine available elsewhere for the essential function which it performs. To discover whether bromine is sufficiently similar in its biological activity actually to replace chlorine in some functions, two experiments were conducted.

In experiment 4, four pairs of cultures, each containing three tomato plants, were prepared. To the first pair (set A) 13 μg-atoms per culture of radioactive chlorine were added; to the second pair (set B) 80 μg-atoms per culture were added; to sets C and D 154 μg-atoms per culture. After growing for 4 weeks, the plants in set A had developed symptoms of chlorine deficiency. At this time the plants were removed from the radioactive culture solutions, the roots rinsed in distilled water, and the plants then transferred to fresh control M6+(m7—Cl) nutrient solutions. To one beaker of each of pairs A–D, 400 μg-atoms per culture of bromine were now

added. In set A, the chlorine-deficient plants which received bromine soon showed recovery, and the new growth was healthy and free from deficiency symptoms. A comparison of the new terminal growth on the two cultures in set A, after addition of bromine to one, is shown in Plate 1, Figure 3.

Two weeks after the addition of bromine, all plants were harvested, and dry weights and total radioactivity in the roots were measured. Harvest data are presented in Table 3. It may be seen that the added bromine caused an appreciable amount of chlorine to be displaced from the plant roots, concurrent with the growth recovery of the tops in the lowest chlorine-level plants of set A.

TABLE 4
EFFECT OF BROMINE ON EXUDATION OF ^{36}Cl FROM DECAPITATED TOMATO PLANTS IN PRESENCE OF
RELATIVELY LARGE AMOUNTS OF OTHER ANIONS

Set	³⁶ Cl before Cutting (μg-atoms)	Chlorine- deficiency Symptoms on Leaves	Bromine Added* (μg-atoms)	Sap Exuded in 24 Hr		Roots		
				Radioactivity (counts/min)		Volume (ml)	Dry Wt. (g)	Residual Activity (counts/min)
				10 Ml	Total			
A	13	Bronzing	—	28	137	49	2.03	4,390
	13	Bronzing	400	122	634	52	1.70	3,740
B	13	Bronzing	—	33	221	67	1.73	5,320
	13	Bronzing	400	84	900	107	2.13	4,150
C	40	None	—	104	593	57	2.50	15,060
	40	None	400	522	3290	63	2.30	12,300
D	40	None	—	58	284	49	2.70	15,500
	40	None	400	566	4410	78	2.40	11,360

* Ratio of added bromide ions to total (nitrate+sulphate+phosphate) ions present in culture solution was approximately 1 : 160.

This trial did not show whether the chlorine was merely exchanged to the culture solution or whether it was displaced upward into the plant; nor did it demonstrate whether the bromine was much more effective in displacing chlorine than were the other anions present in the culture solution. To clarify these points, this trial was repeated in experiment 5, with some modifications. Four pairs of cultures were again transplanted. This time, two pairs (sets A and B) were given 13 $\mu\text{g-atoms}$ of ^{36}Cl per culture, and sets B and C 40 $\mu\text{g-atoms}$ of ^{36}Cl per culture. After 4 weeks growth, the plants were again transferred to M6+(m7—Cl) solutions, but this time the tops were cut off before the addition of 400 $\mu\text{g-atoms}$ Br to one culture from each pair. Rubber tubes were immediately slipped over the cut stems and the exuding sap caught in conical flasks. This exudate was later made slightly alkaline with NaOH and

evaporated to dryness in nickel-plated planchets. The ^{36}Cl displaced from the roots and present in the exuded sap was then counted. The results of this experiment are recorded in Table 4.

Although the added bromine comprised less than 1 per cent. of the total anions present in the culture solution, nevertheless, it increased the concentration of displaced chlorine in the exuding sap by 3–9 times. As in the previous trial with entire plants, the residual activity in the roots was decreased by the addition of bromine to the culture. This decrease, at least at the $40\text{ }\mu\text{g-atoms }^{36}\text{Cl}$ level, was approximately equal to the additional chlorine displaced to the exudate by the bromine. Hence it seems that bromine has a differential power of replacement for chlorine in plant roots and that the displaced chlorine moves upwards through the plant rather than merely being exchanged for bromine in the culture solution.

IV. DISCUSSION

(a) *Yield Effects*

The results from the foregoing experiments demonstrate that chlorine participates in some function essential to the growth of plants. In this way they confirm the results of Broyer *et al.* (1954), Ulrich and Ohki (1956), and Johnson *et al.* (1957). Where the supply of chlorine is severely limiting bromine is able to substitute partially for chlorine although it cannot completely replace it in the tomato plant or subterranean clover, as evidenced by yields being smaller from the cultures receiving added bromine than were obtained by supplying ample chlorine. Also, plants cultured with bromine added instead of chlorine may appear somewhat less healthy, may wilt under conditions of water stress, and may have modified root development. Even when the yield of low-chlorine plants was increased several times by the addition of bromine it was still not possible to show that the added bromine accounted for more than 30–60 per cent. of the total plant yield. The remainder could have been produced by the chlorine acquired during growth from contamination sources.

The possibility that the bromine effects were due to some essential function performed only by bromine, and not chlorine, could not be substantiated. In the three trials in which bromine was supplied to plants having adequate chlorine, no yield increase from the addition of bromine was obtained.

(b) *Ion Relationships*

The finding that chlorine can be largely replaced by another element is reminiscent of the sparing effects of sodium for potassium and strontium for calcium. Chlorine and bromine are alike in many properties as is shown by their similar chemistry and their ionic radii (1.31 and 1.95Å respectively). The bonds retaining chlorine in plant tissue are apparently only of a loose nature and easily broken. Such loose bonds may be much less specific in their requirements than the strong intimate linkages formed by say, nitrogen or carbon in plant tissue.

When bromine does replace chlorine in a complex normally formed with the chloride ion, then, due to the greater mass, larger size, and less intense surface charge of the bromide ion, the similar complex formed with bromine might be expected to form less readily and be less active. In Figure 3. the concentrations of bromine in

plant tissues required to give the maximum growth effect are 2-3 times as great as the concentrations of chlorine required to give equivalent yields thereof. Biological systems are often very specific with regard to the elements which serve particular chemical reactions and, at least in the animal body, the function of chlorine does not appear to be replaceable by bromine.

In the trial with decapitated plants, the roots containing ^{36}Cl , a direct demonstration was given of the ability of bromine to replace chlorine in living tissue. As relatively small numbers of bromide ions displaced far more ^{36}Cl than did larger numbers of nitrate, sulphate, and phosphate ions, it seems that chlorine is held by bonds with specific requirements only satisfied by an ion very similar to the chloride ion. Chlorine displaced from plant roots by bromine apparently moves upwards into the shoots and becomes available to other tissues. Thus the recovery of chlorine-deficient plants when they were given bromine may be largely through the displacement of chlorine from non-essential locations to more vital sites: either at the plant level, as from roots to the young growing shoots; or at the cellular level, as from inactive adsorption sites in the protoplasm to active complexes in the cytoplasm.

An interesting comparison may be drawn between the effects of bromine in displacing chlorine, and the effects of sodium in displacing potassium. The sparing effect of sodium on potassium is well recognized. Several workers, such as Holt and Volk (1945), have obtained large growth effects by applying sodium to potassium-deficient plants. A displacement of potassium by sodium from petioles to leaf blades of potassium-deficient sugar-beets may be inferred from the recent work of Ulrich (1956). Presumably, in the laminae the potassium performs a more specific role. Potassium, like chlorine, apparently forms only loose bonds in plant tissues.

(c) *Plant Functions*

Chlorine has been suggested as necessary for at least two plant processes. Warburg and Luttgens (1946) proposed chlorine as a coenzyme in photosynthesis. In similar experiments of Arnon and Whatley (1949) on the photochemical activity of isolated chloroplasts, bromine was found to exert the same sort of activating effect as chlorine, but to a somewhat lesser extent. A second function for chlorine is suggested in the earlier physiological work on chlorine deficiency (Broyer *et al.* 1954; Johnson *et al.* 1957). Here, localized wilting in leaf blades has been observed as a general symptom of this deficiency in most species studied. The wilted marginal areas of the leaves showed a characteristic recovery when the transpiration stress was removed. From this it may be inferred that chlorine plays some role in the water relations of leaves. Struckmeyer and Wedin (1955) found poor differentiation of xylem in low-chlorine tobacco plants. However, Johnson *et al.* (1957) mention that chlorine-deficient and chlorine-sufficient tomato leaves of the same age, size, and position had the same general histological structure at the time of onset of chlorine-deficiency symptoms in the low-chlorine leaves. There was no obvious difference in xylem development.

V. ACKNOWLEDGMENT

The authors desire to acknowledge the assistance of R. P. Huston during the progress of the work.

CHLORINE AND BROMINE IN PLANT NUTRITION





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EXPLANATION OF PLATE I

- Fig. 1.—Comparative growth of subterranean clover plants cultivated under different halide-supply conditions. 0, no added halide; CL, 400 μ g-atoms of added chlorine per culture; BR, 400 μ g-atoms of added bromine per culture; CL+BR, 400 μ g-atoms each of added chlorine and bromine per culture.
- Fig. 2.—Comparative development of roots from the subterranean clover plants illustrated in Plate 1, Figure 1.
- Fig. 3.—Effect of adding bromine to the culture solution on new growth of tomato shoots, following chlorine deficiency. 400 μ g-atoms of bromine were added to the culture represented on the right, following transfer of each plant from deficiency levels of 13 μ g-atoms of chlorine per culture to "chlorine-free" solutions.

SULPHUR ANALOGUES OF INDOLEACETIC ACID: SYNTHESIS AND BIOLOGICAL ACTIVITY OF SOME THIONAPHTHENACETIC ACIDS

By N. P. KEFFORD* and JUDITH M. KELSO†

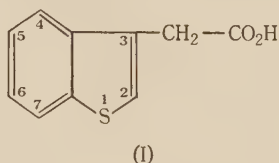
[Manuscript received September 28, 1956]

Summary

The thionaphthen-2-, -3-, -5-, -6-, and -7-acetic acids have been synthesized from the chlorides of the corresponding carboxylic acids by the Arndt-Eistert reaction and their plant growth-regulating activities, as indicated by their effects upon protoplasmic streaming, have been compared with those of indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). The thionaphthen-3- and -7-acetic acids and IAA have similar effects upon protoplasmic streaming, while the effects of the thionaphthen-2-, -5-, and -6-acetic acids differ from those of IAA and resemble in part those of 2, 4-D.

I. INTRODUCTION

Since thionaphthen-3-acetic acid (3-TAA) (I) was found to be an auxin (Crook, Davies, and Smith 1937) attempts have been made to prepare and test biologically



all the isomeric thionaphthenacetic acids. This has been done with the exception of thionaphthen-4-acetic acid (4-TAA), which was synthesized and shown to be an auxin by Kloetzel, Little, and Frisch (1953).

II. MATERIALS AND METHODS

Thionaphthen-3-acetic acid was prepared from the acid chloride of thionaphthen-3-carboxylic acid by Crook and Davies (1937) using the Arndt-Eistert reaction (Bachmann and Struve 1942). Thionaphthen-2-carboxylic acid was similarly converted into thionaphthen-2-acetic acid (2-TAA) (Blicke and Sheets 1948); this had previously been prepared by Crook and Davies (1937) by hydrolysis of the product of condensation of thionaphthen and ethyl bromoacetate, but its structure had not been established. The present paper describes the conversion of the thionaphthen-2-, -5-, -6-, and -7-carboxylic acids into the corresponding acetic acids by means of the Arndt-Eistert reaction.

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The 2-TAA and 3-TAA, synthesized by Crook and Davies (1937), were tested for auxin activity (Crook, Davies, and Smith 1937) using the *Avena* coleoptile curvature test and the split pea epicotyl curvature test, and their activities were compared with those of indole-3-acetic acid (IAA). 3-TAA had about one-thirtieth of the activity of IAA in both tests. 2-TAA had approximately the same activity as 3-TAA in the pea test but was inactive in the *Avena* test. In the present investigation, the effects of the thionaphthen-2-, -3-, -5-, -6-, and -7-acetic acids upon protoplasmic streaming in the cells of the staminal hairs of *Tradescantia virginiana* L. were

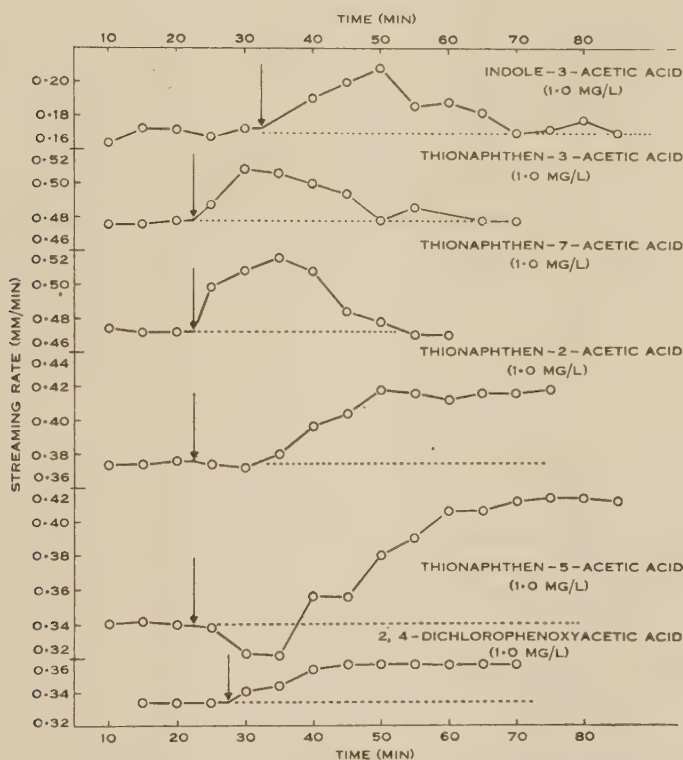


Fig. 1.—Effects of indole-3-acetic acid, the thionaphthenacetic acids, and 2,4-dichlorophenoxyacetic acid upon the protoplasmic streaming rates when added at the times indicated by the arrows. The broken lines extrapolate the basal rates in water, determined before the addition of the acids.

measured, using the method of Kelso and Turner (1955), and the effects were compared with those of the auxins IAA and 2,4-dichlorophenoxyacetic acid (2,4-D). The measurement of protoplasmic streaming rates is not an established method of assay for plant growth-regulating activity, but, as Veldstra (1953) has recommended for such assays, the compounds have been tested over a range of concentrations and the effects compared with those of compounds of known growth-regulating activity when tested under the same conditions.

III. EXPERIMENTAL

(a) *Preparation of the Thionaphthenacetic Acids*.—This was the same process in all cases. The thionaphthencarboxylic acid (0.45 g) was converted to the acid chloride by thionyl chloride (0.84 ml), the excess of which was removed by the addition of dry benzene and distillation under reduced pressure. The residual acid chloride was dissolved in dry ether (5 ml) and gradually added to a cooled, dried solution of diazomethane (from nitrosomethylurea (1.2 g)) in ether. After 12 hr at room temperature the ether was removed and to the residual thionaphthoyldiazo ketone, dissolved in ethanol (5 ml) at about 60°C, a slurry of silver oxide (prepared from 2 ml of a 10% silver nitrate solution) with ethanol (2 ml) was added gradually (1 hr) with shaking. The mixture was then refluxed for 10 min, a little charcoal added, and the filtrate refluxed for 1½ hr with potassium hydroxide (0.2 g). Removal of the alcohol was followed by acidification and the black oil was crystallized several times from boiling water (charcoal). The yield of pure acid was about 20%, but the yield of crude, brown, solid acid was about 60%. The acids are very soluble in cold alcohol, slightly in cold water. The intermediate formation of the ester from the thionaphthoyldiazo ketone gave a better yield than direct conversion to the acid by an aqueous suspension of silver oxide.

The acids which were previously synthesized by Crook and Davies (1937) and have been resynthesized are thionaphthen-2-acetic acid, m.p. 141°C, acid equivalent 194, and thionaphthen-3-acetic acid, m.p. 109°C. The new compounds are: *thionaphthen-5-acetic acid*, m.p. 119°C, acid equivalent 193.5; *thionaphthen-6-acetic acid*, m.p. 86°C, acid equivalent 193.5; and *thionaphthen-7-acetic acid*, m.p. 119°C, acid equivalent 195.0. The theoretical acid equivalent for a thionaphthen-acetic acid is 192.

IV. BIOLOGICAL TESTING

An examination was made of the effects of the above acids, IAA, and 2,4-D on protoplasmic streaming in the staminal hairs of *T. virginiana*, over a concentration range of 0.1–500 mg per litre of distilled water, the pH being adjusted to 4.0. Details of the procedure have been given by Kelso and Turner (1955). All of the acids affected the protoplasmic streaming rate and the particular effects at the 1.0 mg/l level can be compared in Figure 1, where changes in protoplasmic streaming rates for periods of about 1 hr after treatment are shown. IAA at concentrations of 50 and 100 mg/l produced an immediate fall in streaming rate, but the initial rate was restored after about 1 hr, and at concentrations of 0.1–10.0 mg/l there was an immediate promotion of streaming rate, the amount depending upon concentration, with a return to the initial rate after about 1 hr. The same description applies to the effects obtained with 3-TAA and 7-TAA. The activities in protoplasmic streaming of these acids can be compared with that of IAA by expressing the *total effect* (see Thimann and Sweeney 1937) of a given acid concentration as the area under the rate-time curves of protoplasmic streaming, using the rate in water as the base line. This has been done for 3-TAA, 7-TAA, and IAA in Figure 2. It is apparent that the three acids have approximately the same activity in protoplasmic streaming, with maximum promotion at 1.0 mg/l.

As may be seen in Figure 1, 2-, 5-, and 6-TAA differed from IAA in their effects upon protoplasmic streaming rate (the curve for 6-TAA, which is not included in Fig. 1, was the same as that for the -5- acid). The promotion of streaming rate was not immediate on treatment but was preceded by a period of inhibition or of stationary rate, and when an enhanced rate was achieved it was maintained with no sign of a return to the initial rate. This maintenance of a high streaming rate following treat-

ment bears similarity to the effects of 2, 4-D (Fig. 1); this substance, however, produces an immediate promotion of streaming rate. The effects of 2-TAA approach closest to those of 2, 4-D.

Similar data on the auxin activities of all the possible indole acetic acids is not recorded in the literature. This is surprising in view of the large number and variety of compounds that have been tested and the considerable bearing that the auxin activities of the indole acetic acids, other than IAA might have on theories of the mechanism

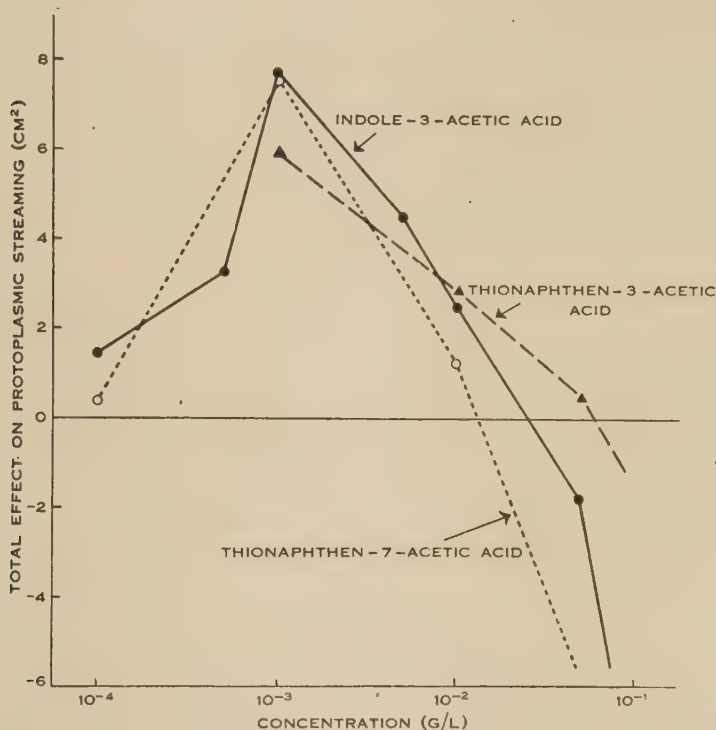


Fig. 2.—Total effects of various concentrations of indole-3-acetic acid, thionaphthen-3-acetic acid, and thionaphthen-7-acetic acid on the protoplasmic streaming rates. The points represent the areas under rate-time curves, such as in Figure 1, using the extrapolated rate in water as base line. Points for the total effects at the highest concentrations used are beyond the axes shown.

of auxin action. However, by analogy with the naphthalene acetic acids, one would expect the 3-, 4-, and 7-TAA to have growth-regulating activity and evidence for this has been produced for the 3- and 7-acids, and the 4-acid had previously been shown to be an auxin (Kloetzel, Little, and Frisch 1953).

V. ACKNOWLEDGMENTS

Thanks are due to Professor W. Davies and Professor J. S. Turner. The former initiated this investigation and directed the chemical work. Partial support was obtained from a grant by Australian Paper Manufacturers Limited.

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STUDIES IN THE PHYSIOLOGY OF *CLOSTRIDIUM BOTULINUM* TYPE E

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[Manuscript received July 26, 1956]

Summary

Ten strains of *Clostridium botulinum* type E have been studied at 12 temperatures between 2.5 and 45°C. Growth proceeded consistently from spore inocula at temperatures between 5 and 37.5°C, but some strains developed slightly at temperatures up to 45°C. Maximum rates of growth occurred at 35°C. The upper and lower temperature limits were several degrees lower than for type A and B strains.

The heat resistance of the spores was about 1/1000th that of spores of some type A and B strains. Type E toxin was found in greatest amount in cultures grown at 25°C. The toxin was most stable at pH 4.5-5 but more heat labile than type A toxin.

Two of 22 samples of marine mud yielded cultures containing *botulinum* toxin. The possible marine origin of type E strains is discussed.

I. INTRODUCTION

Although the physiology of *Clostridium botulinum* has been studied extensively, most of the work has been done with strains of types A and B. Relatively little attention has been given to type E strains which, although not as common as types A and B, have, nevertheless, caused a number of fatal cases of human botulism. The epidemiology of type E botulism has been reviewed by Dolman and Chang (1953) who have shown that, with one exception, all cases were associated with fish or marine mammals. The present paper reports some physiological studies made on 10 type E strains and shows that they constitute a homogeneous group differing in several respects from strains of types A and B.

II. METHODS

The techniques and media (neopeptone-yeast extract-glucose (N.Y.G.)) used were the same as those described by Ohye and Scott (1953). The organisms were grown in evacuated sealed tubes and growth was measured nephelometrically. Some experiments were also made in cooked meat medium (C.M.M.). In view of the much lower heat resistance of type E spores possible non-sporing contaminants were eliminated by heating the sealed tubes for 15 min at 60°C rather than 10 min at 80°C. Each μA of the nephelometer reading was equivalent to approximately $5.5 \pm 0.3 \times 10^{-2} \mu g$ dry weight or $5.1 \pm 0.55 \times 10^4$ cells. According to these values, the type E cells have about three times the light scattering capacity of cells of types A and B, but this may be due to short chains of the type E organisms being counted incorrectly as single cells.

Heat resistance of the spores was determined by the methods of Olsen and Scott (1950) using tubes of pork infusion thioglycollate starch agar incubated for 2 weeks at 25°C. The heat stability of the toxin was determined by methods described by Scott and Stewart (1950).

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The 10 strains used were numbered 101 to 110 in this Laboratory. Strains 101 and 102 were isolated by Dr. Elizabeth Hazen, 101 (35396) from canned sprats, and 102 (36208) from smoked salmon (Hazen 1937, 1938). Strains 103 and 104 were isolated by Prof. C. E. Dolman, being respectively his Nanaimo (Dolman and Kerr 1947) and Vancouver herring strains (Dolman *et al.* 1950). Strains 105–110 were originally isolated in U.S.S.R. and two of them were described by Gunnison, Cummings, and Meyer (1936). These were received as strains 7–4, 2, 8, 151, 30–6, and 30–17 respectively. Strain 102 was obtained from Dr. Hazen, strains 103 and 104 from Prof. Dolman, and the remaining 7 strains from Prof. K. F. Meyer.

III. RESULTS

(a) Temperature Relations

(i) *Rates of Growth*.—Although all 10 strains were studied at 12 temperatures from 2.5 to 45°C complete results were obtained for only nine strains. Spores of strain

TABLE 1
MEAN RATES OF GROWTH AND THEIR STANDARD ERROR AT VARIOUS TEMPERATURES IN N.Y.G.
MEDIUM

Temperature (°C)	No. of Strains	Mean Rate (divisions/hr)	S.E. of Mean
5.0	9	0.0234	0.0019
10.0	9	0.139	0.0084
15.0	10	0.326	0.0078
20.0	9	0.539	0.0198
25.0	10	1.006	0.0251
30.0	9	1.492	0.0379
35.0	10	1.777	0.0497
37.5	9	1.456	0.1221
40.0	9	1.196	0.2307

101 often failed to initiate growth, and when growth of this strain was observed the lag period was usually much greater than for the other strains. Although the observed rates of growth of strain 101 were very similar to the other strains at several temperatures, there is no certainty that it does not differ from the others, and subsequent discussion will, therefore, be limited to the remaining nine cultures.

In N.Y.G. medium all strains developed from a spore inoculum at temperatures between 10 and 37.5°C. Growth at 37.5°C was often delayed for 1–3 days. At 5°C only six strains grew from a spore inoculum, but all strains grew when growth was initiated at 10°C. Similarly at 40°C only three strains developed from a spore inoculum, whereas all strains grew when growth commenced at 30°C. The mean rates of growth and their standard errors are given in Table 1, the data for 5, 37.5, and 40°C being based on cultures transferred from intermediate temperatures. Variability of the data, expressed as a percentage of the mean, is least at temperatures between 15 and 35°C.

The mean rates of growth and their temperature coefficient are shown also in Figure 1 together with the rates previously reported for types A and B strains. For the type E strains the maximum rate of growth occurs at *c.* 35°C compared with

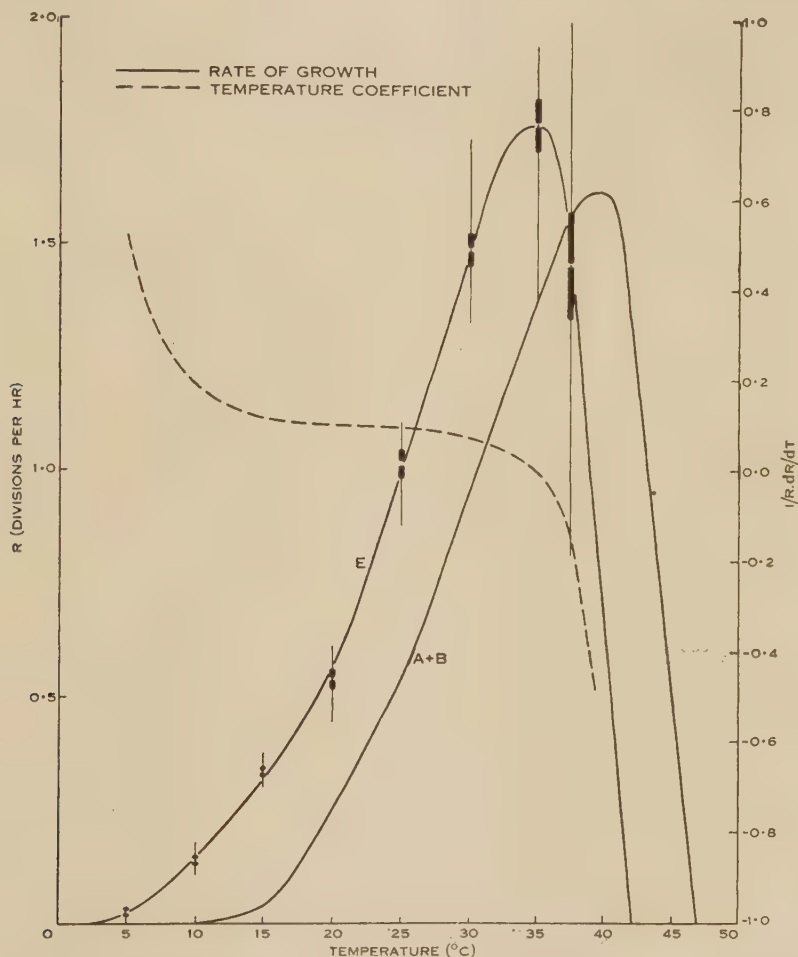


Fig. 1.—Relation between the mean rate of growth (R), the temperature coefficient of R , and the temperature (t) in N.Y.G. medium. The heavy vertical lines show the mean value of $R \pm$ its standard error; the light vertical lines show the range of values observed at each temperature. The curve A and B shows the mean values of R previously obtained for types A and B strains (Ohye and Scott 1953).

40°C for A and B strains. It will be noted that the curves intersect at about 37°C, and so result in a fortuitous equality of growth rates at this temperature. It is clear that the type E strains have minimum temperatures for growth 8–10°C below the minimum for types A and B and that the maximum temperatures are also about 5°C less. At several intermediate temperatures a temperature difference of 5–7°C suffices to equalize the rates for types A, B, and E strains.

As shown previously for types A and B strains, the temperature coefficient for type E strains also becomes large at low temperatures. The rates of growth are changed by about 10 per cent. per °C at 25°C, by 20 per cent. at 10°C, and by 50 per cent. at 5°C.

(ii) *Lag*.—The deduced lag periods, which include the time required for spore germination, varied widely with temperature. They were, however, subject to considerable variation at any one temperature and for the most part, differences

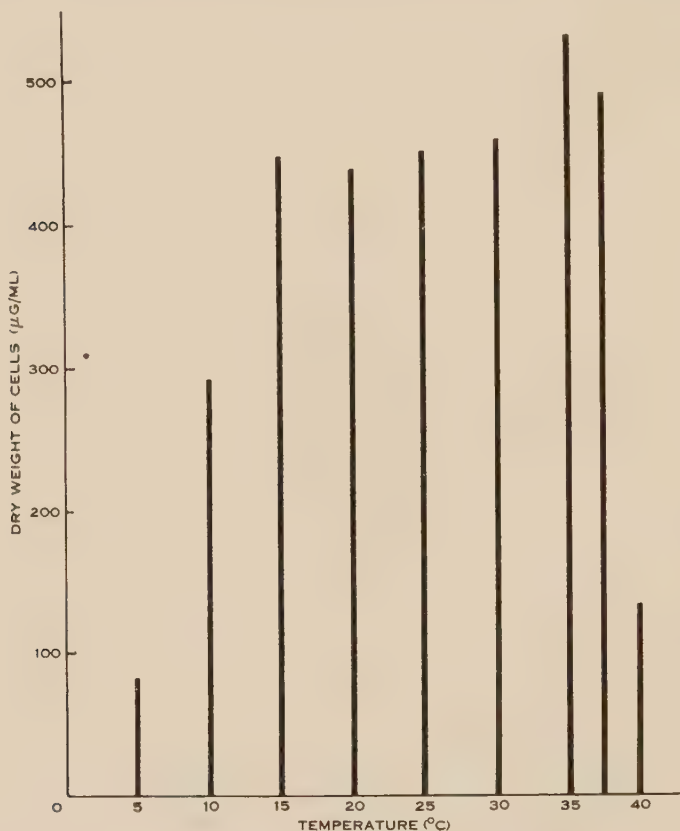


Fig. 2.—Relation between the yield of cells and temperature (N.Y.G. medium).

between strains were not consistent. From 20 to 35°C the mean lag period was about 4 hr with variations from 1 to 10 hr. There were no consistent differences between temperatures within this range. At 10 and 15°C the lag period fluctuated between 9 and 33 hr, the mean value being about 24 hr. At 37.5°C the lag varied greatly between 1–2 hr and 2–3 days.

(iii) *Yield of Cells*.—The maximum yield of cells showed a considerable dependence on temperature as has been reported previously for types A and B strains. The average results for all experiments are shown in Figure 2. The maximum density of the cultures did not vary greatly between 15 and 37.5°C but diminished greatly at temperatures outside this range.

(iv) *Lower Temperature Limits.*—Although only six strains grew from spore inocula in N.Y.G. medium at 5°C all strains grew in C.M.M. at the same temperature. Gas production, sufficient to lift the cooked meat, was evident 3–4 weeks after inoculation. When C.M.M. cultures were opened after 8 weeks at 5°C, toxin lethal to mice was found in four cultures, the titre being of the order of 10 mouse-doses per ml. The remaining five cultures did not kill mice after the injection of 0.2 ml, but some of these presumably contained a little toxin as the mice showed transient symptoms of botulism from which they recovered.

Duplicate tubes of all 10 strains were incubated in N.Y.G. medium at 2.5°C for 22 weeks. There was no change in turbidity, and after heating at 60°C for 30 min the

TABLE 2

VIALE COUNTS AND pH VALUES OF C.M.M. CULTURES HELD AT 2.5°C FOR 20 WEEKS AND AT 45°C FOR 1 WEEK

Strain	2.5°C			45°C		
	pH	Viable Count/Ml		pH	Viable Count/Ml	
		Unheated	Heated*		Unheated	Heated*
101	6.78	830	570			
102	6.76	<10	<10	6.51	5000	<1
103	6.75	550	700	6.70	7000	<1
104	6.76	900	650	7.17	1120	350
105	6.85	630	820	7.20	830	800
106	6.80	770	930	7.22	940	800
107	6.76	840	650	7.22	540	500
108	6.86	430	500	7.24	820	960
109	6.80	<10	<10	7.20	500	<1
110	6.76	650	600	7.20	170	<1

* Inoculum heated for 15 min at 60°C.

sealed tubes were incubated at 25°C. Growth occurred in all tubes within 24 hr. There is evidence, therefore, that the spores did not germinate and that the medium remained suitable for growth. Similar experiments were carried out in sealed tubes of C.M.M. incubated at 2.5°C for 20 weeks. At the end of this time all tubes were opened. There was no macroscopic or microscopic evidence of growth and when 0.4-ml aliquots of each tube were injected into mice, there were no deaths and none of the mice showed symptoms of botulism during observation for 3 days. Portions of the fluid were taken and 0.1-ml aliquots inoculated, unheated and heated, into tubes of pork infusion thioglycollate starch agar. Results are shown in Table 2. The remaining portion of each C.M.M. tube was transferred to 25°C and growth occurred in all tubes within 24 hr. For seven of the strains, therefore, the evidence is that the spores did not germinate within 5 months at 2.5°C, but for strains 102 and 109, the original inoculum had become largely non-viable.

(v) *Upper Temperature Limits.*—In three series of experiments, only strains 103, 104, and 110 developed in N.Y.G. medium within 5–7 days at 40°C. Strain 103 grew on two occasions and 109 and 110 on only one occasion. In all tubes in which growth did not occur the inoculum remained stable to 15 min at 60°C and growth ensued after transfer to 25°C. When N.Y.G. cultures were transferred from 30 to 40°C all strains grew, but the maximum turbidity for strains 103 and 104 was much greater than for the other seven strains. When N.Y.G. cultures were transferred from 30 to 42.5°C sustained growth occurred only with strains 102, 103, and 104. For the other six strains there was only a small and transient rise in turbidity which at no time was more than four times the turbidity at the time of transfer. When transferred from 30°C the cultures had turbidities equivalent to cell populations of $1-5 \times 10^6$ /ml.

When C.M.M. cultures were inoculated with spores and held for 7 days at 45°C, slight growth occurred with strains 102 and 103 and the inoculum of 109 and 110 became heat labile. With strain 104, about 70 per cent. of the spores became heat labile. With the other strains the spores did not germinate, as judged by viable counts (Table 2) on heated and unheated aliquots of the cultures. Cultures of 102 and 103 showed microscopic evidence of slight growth, a few irregularly stained rods being found in smears. No organisms were seen in stained smears from the other cultures. None of the cultures contained demonstrable amounts of toxin when 0.4-ml aliquots were injected into mice.

The results indicate that growth is unlikely to proceed at temperatures greater than 45°C and that about half the strains are unable to grow at this temperature. There is evidence of some consistent differences between strains, and strains 102, 103, 104, 109, and 110 appear to have maximum temperatures slightly greater than strains 105, 106, 107, and 108.

(b) *Sporulation and Heat Resistance of Spores*

Sporulation varied greatly with temperature and the medium in which the organisms were grown. At 25 and 30°C the yield of spores was much greater than at 37 and 20°C. A number of media including N.Y.G., peptic and papain digests of ox heart, and casitone and peptone solutions, each containing 0.01 per cent. sodium thioglycollate, were tested with and without meat particles at pH levels of 6.0, 7.5, and 9.0. Sporulation was enhanced by the presence of meat particles in all media, the best yields being obtained in papain-digest media with meat particles and an initial pH of 7.5. In this medium as many as 90–95 per cent. of the cells formed spores in 5 days at 30°C. The addition of 0.5 per cent. glucose to this medium greatly increased the yield of cells, but seriously depressed sporulation.

In contrast to types A and B cultures in which motility was readily observed only in young cultures, active motility was well retained in old type E cultures. It was, in fact, common to observe active motility in cells which had already formed a mature, refractile sporangium.

Although it is generally agreed that type E spores are much more heat labile than spores of types A and B strains, quantitative data on rates of destruction of type E spores have not yet been published.

Spores of strains 103 and 108 were heated in M/15 phosphate buffer pH 7.0 and the viable spores enumerated after various periods of heating at 70, 75, and 80°C. The logarithm of the number of viable spores was approximately a linear function of time at all three temperatures and the following decimal reduction times indicate the slopes of the curves. For strain 103 these were 36, 10, and 3.3 min and, for strain 108, 7.8, 3.1, and 0.4 min at 70, 75, and 80°C respectively. The temperature coefficient is of the same order as for data on types A and B spores. The results show that strain 103 is somewhat more heat resistant than strain 108, but illustrate clearly the very low resistance in comparison with types A and B strains.

For strain 103, for instance, a Q_{10} of 10 indicates a decimal reduction time of 2 sec at 100°C. For some type A and B strains, the data of Olsen and Scott (1950) show these to be over 1000 times more resistant at 100°C. As would be expected from the above results the viable counts of type E spores were virtually unaffected by heating for 15–30 min at 60°C.

(c) *Toxin Production and Heat Resistance of the Toxin*

Mice injected with type E toxin showed, relative to others receiving type A or B toxins, certain differences in the toxic syndrome. While the symptoms of respiratory distress and paralysis were rather similar they had a more rapid onset with type E toxin. Mice receiving more than 10 lethal doses usually showed the first symptoms within 2–4 hr and, indeed, death often occurred within 4 hr. It was unusual for a mouse to die if the initial symptoms were not apparent 6 hr after injection. Almost all mice receiving a lethal dose of type E toxin died within 24 hr of injection, whereas with A and B toxins deaths 2–4 days after injection were not uncommon. Mice showing symptoms of botulism 24 hr after injection of type E toxin almost always recovered and symptoms disappeared within a further 24 hr. Therefore, mice were not observed for more than 3 days after injection.

Several strains produced toxin in C.M.M. within 7 days at 25°C with titres of 1000–5000 mouse-doses per ml. At 30 and 37°C titres were lower and, on occasions, cultures grown at 37°C were non-toxic. In contrast to the effect with type A cultures, the addition of 0.5 per cent. glucose did not enhance the titre, but rather tended to reduce it.

Attempts to concentrate the toxin by acid precipitation were unsuccessful. When C.M.M. cultures of strain 101 were brought to pH 5 with HCl a precipitate formed, but when this was redissolved in phosphate buffer of pH 7 the fluid was no more toxic than the original fluid. Further reduction of the pH to 3.5, 2.5, and 1.5 caused no reduction in the titre.

The heat stability of toxin produced by strain 101 has been examined in several experiments. The toxin is appreciably less resistant than type A toxin, but as with the latter, has its greatest stability at pH 4.5–5. Some typical results are shown in Table 3, in which it may be seen that toxin was destroyed within 5 min at 60°C at pH 7.5 and 3.5 but persisted with a titre of 4^{-2} after 40 min at pH 4.6 and 4.9. Other experiments also showed greatest stability at pH 4.5–5 when toxin was heated at 65°C, the rate of destruction being 6–10 times greater at the higher temperature.

Attempts to increase the heat stability of type E toxin by heating in solutions of high ionic strength were also unsuccessful. When an equal volume of a solution of mixed electrolytes of ionic strength 2.0 (Scott 1950) was added to toxic fluid from a C.M.M. culture and heated at 60°C at pH 5.0 the stability was not increased in

TABLE 3
DESTRUCTION OF TYPE E (101) TOXIN IN C.M.M. FLUID AT 60°C

The results represent the highest dilution to cause death of mice injected with 0.2 ml. Initial titre 4^{-5} . 0 = no deaths with undiluted toxin, — = not done

pH	Time of Heating (min)			
	5	10	20	40
7.5	0	—	—	—
6.3	4^{-1}	4^{-1}	4^0	—
5.5	—	4^{-2}	4^0	—
5.2	—	4^{-1}	4^0	4^0
4.9	—	4^{-3}	4^{-3}	4^{-2}
4.6	—	4^{-4}	4^{-2}	4^{-2}
4.3	—	4^{-4}	4^{-2}	4^{-1}
4.0	—	4^{-2}	4^0	4^0
3.5	0	—	—	—

comparison with another aliquot mixed with an equal volume of distilled water. In this respect, therefore, type E toxin differs from type A toxin for which heat stability was considerably enhanced in solutions of high ionic strength (Scott 1950).

(d) *Cl. botulinum* in Marine Muds

In view of the lower temperature range for the growth of type E strains it was decided to examine some marine muds for the presence of this organism. Twenty-two samples were collected from Tasmanian and New South Wales coastal waters, generally just a few feet below the low tide level. Samples of 10 g were added to 100 ml of C.M.M., heated at 60°C for 30 min, and incubated at 10°C. Good growth of a variety of *Clostridium* spp. ensued in all cultures, and several of the isolates were found to produce spores which were, like type E spores, readily destroyed at 70 and 75°C. After incubation for 30 days aliquots of 0.2 ml were injected into mice. *Cl. botulinum* toxin was detected in cultures from two muds, both collected at 6–8 fm a few miles from Hobart, Tas. These observations were confirmed in cultures prepared from further aliquots of these muds which had been held at 0°C. When the toxic fluid was mixed with antitoxin 1 hr before injection, the mice were protected by type B, but not by types A or E antitoxins. Repeated efforts to isolate the toxigenic organism, and indeed to prepare toxic subcultures, were uniformly unsuccessful. The indication that type B toxin was present in these cultures was unexpected as Ohye and Scott (1953) had found a number of type B strains were unable to grow at 10°C.

Since these experiments were completed, Pederson (1953) reported an outbreak of type E botulism in Denmark in which marinated herring was the causal food. The initial finding of the State Serum Institute was that the herring contained *botulinum* toxin type B. Subsequently, a pure culture of type E *Cl. botulinum* was isolated, the toxin of which was partially neutralized by type B antitoxin, mice receiving type B antitoxin showing much lower mortality. In the light of these results, therefore, it is still possible that the toxin found in cultures from the two marine muds was, in fact, type E.

Pederson (1955) isolated *Cl. botulinum* type E from 16 out of 19 samples of mud collected from Copenhagen harbour, and from Agluitsk Bay, Greenland, but from only one of 19 soil samples collected around the Copenhagen fish markets. These results indicate the prevalence of type E organisms in marine muds.

IV. DISCUSSION

The main feature of the results is the clear demonstration that the temperature relations of type E strains differ substantially from those for the toxigenically related types A and B strains. As the temperature range of microorganisms is an attribute with great genetic stability there is a strong suggestion that the natural habitat of type E organisms is at a lower average temperature than the natural habitat of A and B strains. The isolation of type E organisms from marine muds by Pederson (1955), together with the characteristic association of type E botulism with marine products, suggests a marine rather than a terrestrial habitat.

The finding that many of the clostridia in marine muds produce spores of low resistance to heat shows that the widespread assumption that bacterial spores survive 10 min at 80°C is unsound. Failure to recognize this is probably the reason why organisms producing heat labile spores have not been reported more frequently. For spores like those of strain 108, 10 min at 80°C would suffice to reduce the number of viable spores by a factor of 10^{25} .

There is general agreement that although type E strains grow readily at 37°C the cultures grown at this temperature are frequently non-toxic. It will be seen from Figure 1 that at 37°C the temperature coefficient is already negative and it would not be surprising if some of the normal properties of the organisms are impaired at this temperature. It is also possible that variations in the temperatures actually realized in incubators nominally operating at 37°C could account for some of the differences between observers (Dolman *et al.* 1950; Barron and Reed 1954; Pederson 1955).

It is to be noted that the strains examined in these experiments have been isolated in Europe and various parts of North America, and that, at least in respect of temperature requirements, they constitute a reasonably uniform group. It is true that some of the strains were consistently somewhat more vigorous in growth at temperatures above 37°C, but when compared with the differences from A and B strains these variations within the type E group were relatively small. Although the 10 strains used in the present study include most of those isolated prior to 1955, further observations on additional strains would help in assessing the variations which may normally occur within the type E group.

V. ACKNOWLEDGMENTS

The authors are indebted to Messrs. A. M. Olsen and E. J. F. Wood, Division of Fisheries, C.S.I.R.O., for the collection of mud samples.

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INCREASED THIOLYCOLLATE EXTRACTION OF WOOL PROTEINS FOLLOWING SUPERCONTRACTION

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[Manuscript received October 5, 1956]

Summary

Supercontraction of wool or of wool cells by heating in 9M LiCl or 8M phenol facilitates the subsequent extraction of the proteins with alkaline 0.1M thioglycollate solutions during 20 min at 50°C. The amount of protein extracted with 0.1M thioglycollate initially at pH 10.0, for example, approximates to that removed from untreated wool with 0.1M thioglycollate initially at pH 10.5. The electrophoresis patterns of the proteins extracted from supercontracted wool also resemble the patterns of extracts of normal wool obtained at higher pH values. This, together with evidence for the absence of amide bond splitting, suggests that fractional extraction with alkaline thioglycollate yields proteins pre-existing in the wool and not formed during extraction. The electrophoresis patterns would therefore appear to reflect the protein composition of reduced wool.

Extraction of supercontracted wool with alkaline thioglycollate allows the wool to revert rapidly to the original uncontracted state having low thioglycollate extractability. Partial reversal of extractability is likewise achieved by prolonging the period of heating in the LiCl solution and complete reversal by heating the LiCl-supercontracted wool in water.

I. INTRODUCTION

It has been shown recently that preheating wool in water for 20 min at temperatures above 70°C reduces the amount of protein extractable with alkaline solutions of potassium thioglycollate (Lennox 1956). This effect reaches a maximum at 120°C, the lowest temperature at which hydrothermal supercontraction occurs. In contrast, it has now been observed that wool which has been markedly supercontracted by heating at 96°C in concentrated aqueous solutions of LiCl (Alexander 1951) or phenol (Elöd and Zahn 1949) yields greater amounts of protein to alkaline thioglycollate than does untreated wool. The quantities of protein extracted after supercontracting the fibres under various conditions and the electrophoretic properties of the extracts are described in the present paper.

II. MATERIALS AND METHODS

(a) Wool

Merino 64's wool top was repeatedly washed at room temperature with diethyl ether and then with water to remove oil and other impurities, and dried in a current of warm air.

(b) Wool Cells

The free cells were prepared by aseptic tryptic digestion of the Merino 64's wool followed by thorough washing and drying.

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(c) *Pretreatment*

Supercontraction was effected by heating wool in 9M LiCl or 8M phenol at 96°C in a boiling water-bath for either 20 or 40 min as indicated. In these pretreatments and in the extraction experiments 30 ml of solution were used per 1 g wool treated unless stated otherwise. After supercontraction the samples were rinsed repeatedly in water and squeezed in dry fabric and absorbent paper to remove free water. The weight of the wool was unchanged by supercontraction. The striking reduction in length of a sample of wool top produced by supercontraction is shown in Figure 1.

To heat the wool samples in water at 110°C they were enclosed in a stainless steel pressure-vessel immersed in an oil-bath. About 1–2 ml of the 30 ml water in the vessel were lost as steam when heating it to the temperature of the bath and before closing the valve.

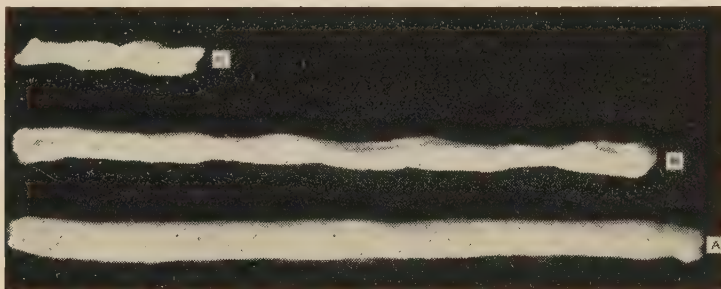


Fig. 1.—Shrinkage of wool top obtained by supercontraction by heating for 40 min at 96°C in 9M LiCl. Individual wool fibres supercontract by only 28 per cent. under these conditions but the bending and twisting of fibres while in the parallel array in wool top cause it to shorten by 75 per cent. A, unheated; B, heated in water; C, heated in 9M LiCl.

(d) *Extraction with Thioglycollate*

The proteins were extracted from wool by heating for 20 min at 50°C in 0.1M thioglycollate adjusted to the desired initial pH value with KOH. The amount of protein extracted was estimated by determining total nitrogen by the micro-Kjeldahl method and applying the conversion factor 6.25.

(e) *Electrophoresis*

Electrophoresis of the wool proteins was carried out as previously described (Gillespie and Lennox 1953, 1955*a*, 1955*b*). The experiments were performed using a buffer of ionic strength 0.22 containing 0.05M thioglycollic acid and glycine at 0.1M concentration, the pH being adjusted to 11.0 with KOH. In most experiments a potential gradient of about 2.5 V/cm was applied for 6 hr at 1°C. As in previous electrophoretic studies on wool proteins extracted with thioglycollate (Gillespie and Lennox 1953, 1955*a*), only the ascending patterns are reported. Anomalous peaks occur in the descending patterns which have been attributed to aggregation (Gillespie and Lennox 1955*b*).

III. RESULTS

(a) *Factors Affecting the Extraction of Proteins from Supercontracted Wool*

(i) *Concentration of LiCl.*—The curve relating thioglycollate solubility with concentration of LiCl (Fig. 2) resembles the concentration–supercontraction curve for this salt (Crewther and Dowling, personal communication), both showing maxima at about 8–9M LiCl.

(ii) *Effect of Previous Treatment of Wool.*—Supercontraction by heating to 96°C for 20 min in 9M LiCl at pH 5 (determined with the hydrogen electrode and by comparison with buffer applied to pH indicator paper) allowed less protein to be extracted from carbonized wool containing residual H_2SO_4 than from fleece wool



Fig. 2.—Effect of concentration of LiCl used for 40 min pretreatment at 96°C on extraction of wool protein during 20 min at 50°C in 0.1M thioglycollate initially at pH 11.0.

which had been freed of wax and suint by extraction with diethyl ether and water. Fleece wool, in turn, yielded less protein to the thioglycollate than did the slightly alkaline wool top (scoured with soap and soda) used in these investigations.

(iii) *pH of Thioglycollate.*—As with normal wool, the quantity of protein extracted with thioglycollate from wool supercontracted in either 9M LiCl or 8M phenol increased with the initial pH of the extractant (Fig. 3), but the quantity extracted at any particular pH over the range 9.5–12 was appreciably greater for the supercontracted wool than for the normal wool.

(iv) *Time of Heating in LiCl.*—The quantity of protein extractable with thioglycollate initially at pH 10.5 increased with the period of preheating in 9M LiCl up to 20 min but thereafter it decreased (Fig. 4).

(v) *Soaking and Drying Supercontracted Wool before Extraction.*—To ascertain whether drying or additional rinsing after supercontraction for 40 min at 96°C in 9M LiCl affected the extractability with thioglycollate, the standard extraction procedure using 0.1M thioglycollate initially at pH 11.0 was applied to several samples

of supercontracted wool which had been subjected to various additional treatments. The first samples were extracted after washing for only 2 min in running water. The second group was dried in a current of warm air before extraction. The third and fourth groups were soaked for an additional 30 and 180 min respectively after rinsing and before extraction to remove any further traces of LiCl. The amount of protein extracted from all these samples was essentially the same, being in the range 45–49 per cent. In a separate experiment, supercontraction by heating for 20 min in 9M LiCl at 96°C was shown to be irreversible even after soaking in distilled water for several weeks.

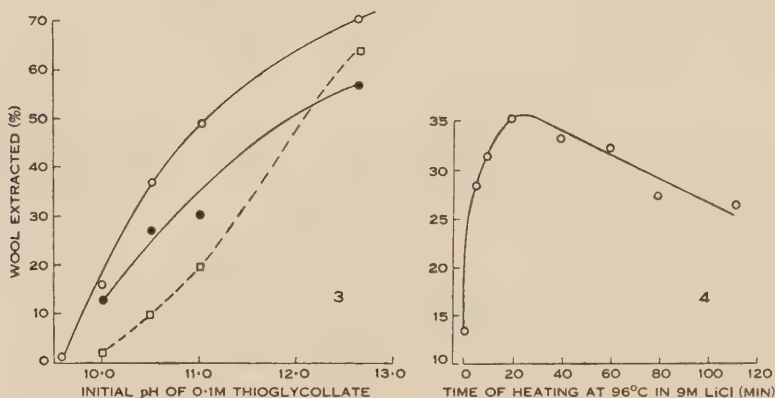


Fig. 3.—Extraction of proteins during 20 min at 50°C with 0.1M thioglycollate at various pH values from wool previously supercontracted by heating for 40 min at 96°C in 9M LiCl (○), or in 8M phenol (●). □ Extraction of untreated wool.

Fig. 4.—Relationship between time of preheating at 96°C in 9M LiCl on subsequent extractability during 20 min at 50°C in 0.1M thioglycollate initially at pH 10.5.

(vi) *Heating in Solutions Containing Both LiCl and Thioglycollate.*—Whereas wool supercontracted in 9M LiCl lost 29 per cent. of its protein during 20 min at 50°C in 0.1M thioglycollate initially at pH 10.5, the addition of 9M LiCl to the thioglycollate solution reduced the extraction to 4 per cent. Heating wool without previous supercontraction in a solution containing 9M LiCl and 0.1M thioglycollate adjusted to pH 10.5 (using indicator paper) extracted no protein, the 9M LiCl apparently repressing the solvent action of the thioglycollate for the wool proteins. In this experiment, wool digestion was measured by washing, drying, and weighing the residue after extraction. The repressive effect of high concentrations of salt on the extraction of proteins with alkaline thioglycollate was confirmed in another experiment in which the concentration of K_2CO_3 in a solution containing 0.1M thioglycollate was increased from 2 to 4M. Although the pH was thereby increased from 10.1 to 10.5 the total protein extracted, as determined by weighing the washed undigested residue, fell from 68 to 17 per cent. In this particular experiment 100 ml of solution per 1 g wool treated were used instead of 30 ml.

(vii) *Reversal of the Effect of Heating in Water or in LiCl Solution.*—In Figure 5 are shown the effects of preheating wool in water and in 9M LiCl on the extractability of its proteins and also the apparent reversal of each of these two effects. The low thioglycollate extractability obtained after heating wool in water at 110°C is seen to be increased to the level of untreated wool by subsequently heating it in 9M LiCl at 96°C. Similarly the enhanced extractability obtained after heating firstly in 9M LiCl is reversed by subsequent heating in water.

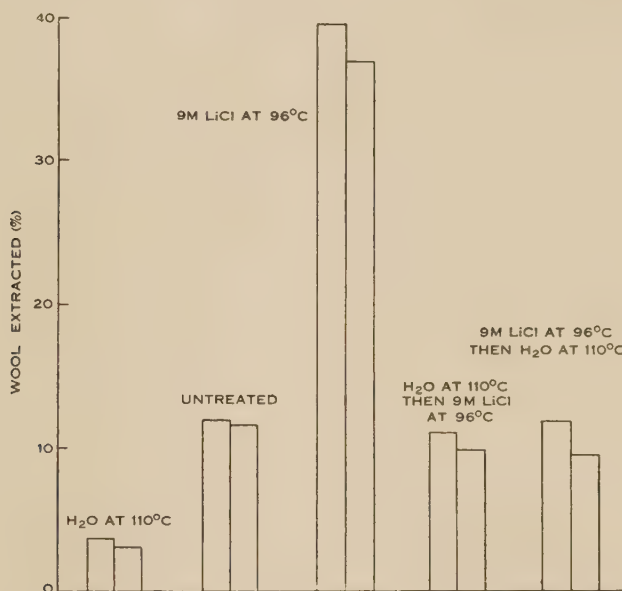


Fig. 5.—Opposite effects of preheating wool for 20 min in water and in 9M LiCl on extraction of protein during 20 min at 50°C in 0.1M thioglycollate (initially at pH 10.5) and the reversal of these effects. Extraction percentages are shown for duplicate experiments.

(viii) *Loss of Supercontraction and of Protein Extractability during Repeated Treatment with Thioglycollate.*—The alkaline thioglycollate fractional extraction method of recovering keratine 2 from wool (Gillespie and Lennox 1953, 1955a), was also applied to normal wool and 9M LiCl-supercontracted wool in the same experiment. This method removed the major portion of the protein from the latter in the first pH 10.5 extraction, in contrast to the normal wool which yielded less than half this amount of protein in the first extraction but a greater amount in the second pH 10.5 extraction (Fig. 6). That the first pH 10.5 extraction of supercontracted wool removed some keratine 2 from the fibres together with other wool proteins is evidenced by the lower content of protein in extract F (Fig. 6), which yields pure keratine 2 in the extraction of normal wool, and also by the presence of the peak corresponding to keratine 2 in the electrophoretic pattern of extract A of supercontracted wool. Extraction of supercontracted wool with alkaline thioglycollate

was complicated by reversal of the supercontracted fibres to their normal length within 3 min of immersion in the 0.1M thioglycollate at pH 10.5. Only the first extraction can therefore be regarded as reflecting the effect of supercontraction. To compensate for the readier extraction of protein from the supercontracted fibres, 0.1M thioglycollate at pH 10.0 was used for the first five extractions in another experiment and the residue was extracted with 0.1M thioglycollate at pH 11.6 (Fig. 6). Although less protein was removed in extract A by 0.1M thioglycollate at pH 10.0 than at pH 10.5, the fibres again reverted to their original length and the protein removed decreased progressively throughout the series.

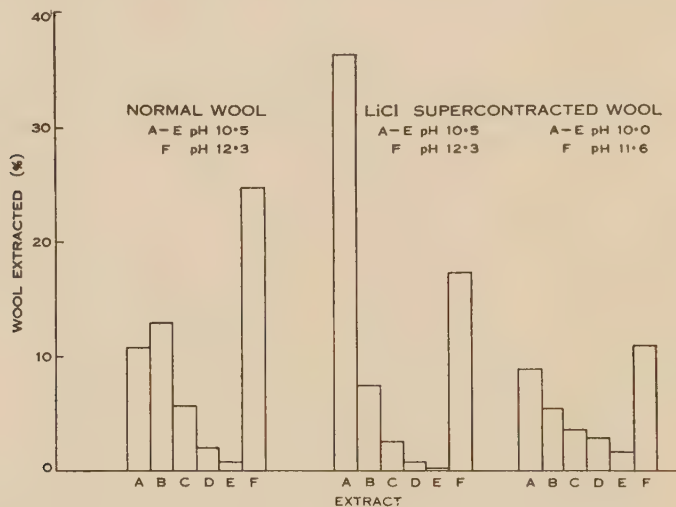


Fig. 6.—Protein extraction of normal and 9M LiCl-supercontracted wool five times with 0.1M thioglycollate initially at pH 10.5 then once with 0.1M thioglycollate initially at pH 12.3, also of supercontracted wool five times with 0.1M thioglycollate at pH 10.0 then once with thioglycollate at pH 11.6.

(ix) *Supercontracted Wool Cells*.—As with the intact fibre, heating wool cells in water at 110°C reduced the extractability of the constituent proteins in alkaline thioglycollate (Lennox 1956) and supercontraction in 9M LiCl at 96°C caused it to increase (Fig. 7). Since more protein is extracted from the untreated wool cells than from intact wool there was less margin for increasing the extraction from the cells by supercontraction than with wool itself.

(b) *Electrophoresis Patterns of Extracts of Normal and Supercontracted Wool*

(i) *Wool Supercontracted in LiCl*.—The electrophoresis patterns of the extracts of normal and LiCl-supercontracted wool, reported in Figure 3 and obtained with 0.1M thioglycollate at the initial pH's 10.0, 10.5, 11.0, and 12.6, are shown in Plate 1. The pH 10.0 and 10.5 extracts were freeze-dried to allow the solutions to be adjusted where possible to 1.5 per cent. protein concentration for electrophoresis; the pH 10.0 extract of normal wool yielded only sufficient protein for a 1.1 per cent. solution.

In the patterns corresponding to the pH 10.0, 10.5, and 11.0 extracts of normal wool, peaks can be seen corresponding to the four components designated kerateines 1, 2, 3, and 4 (from left to right in order of increasing mobility). The main effect of supercontraction appears to have been to allow as much wool protein to be extracted at the low pH values as was recovered from normal wool at a higher pH, and also to yield extracts at the lower pH values having similar electrophoretic patterns to those obtained from normal wool at higher pH. Thus the pattern of the pH 10.5 extract of supercontracted wool resembles more closely the pH 11.0 pattern of normal wool than the pH 10.5 pattern.

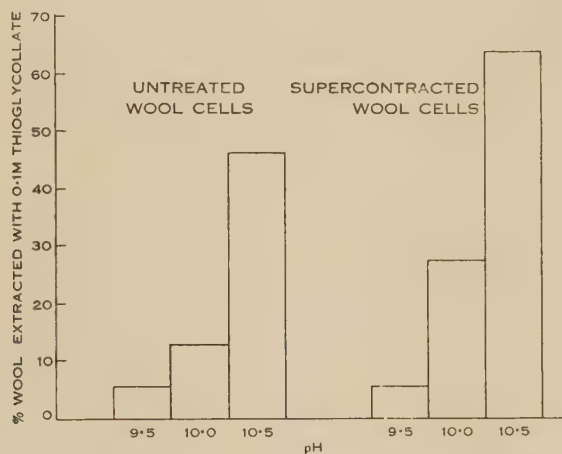


Fig. 7.—Extraction of protein from cortical cells during 20 min at 50°C with 0.1M thioglycollate at various pH values before and after supercontraction by heating for 40 min at 50°C in 9M LiCl. Each extraction was performed on a separate sample of cells.

Although kerateines 1-4 can be readily distinguished in the electrophoresis patterns of extracts of normal wool prepared at pH values 10.0, 10.5, and 11.0 (Plate 1), in the pH 11.0 extract the movement of kerateine 4 was retarded, and in the pH 12.6 extract its apparent mobility was reduced to that of kerateine 3. The peak corresponding to kerateine 3 in extracts prepared at pH 12.6 therefore carries a spike. Just as the pH 10.5 extract of supercontracted wool closely resembles the pH 11.0 extract of normal wool so also does the pH 11.0 extract of supercontracted wool resemble the pH 12.6 extract of normal wool, the movement of the kerateine 4 again being retarded and its spiked peak therefore coinciding in position with the peak of kerateine 3. Retardation of the leading component in the ascending limb with increase in concentration of the other proteins in the mixture is a well-known phenomenon in the electrophoresis of proteins. A similar retardation of kerateine 4 appears to have occurred in the pH 10.0 extract of supercontracted wool. This has been confirmed in two other experiments.

(ii) *Wool Supercontracted in Phenol*.—The patterns of the pH 10.5 and 11.0 0.1M thioglycollate extracts of wool supercontracted in 8M phenol resemble those

of the thioglycollate extracts of the LiCl-supercontracted samples. They exhibit a greater ratio of keratine 2 to the other components than do the patterns of extracts of untreated wool obtained with thioglycollate at the same pH values (Plate 2).

IV. DISCUSSION

In a previous paper (Lennox 1956) the loss in thioglycollate extractability of wool proteins, produced by heating wool in water, was attributed to slight changes in the molecular configuration of the major proteins, enabling the formation of additional hydrogen bonds which oppose solution. In an analogous manner the enhanced extractability of the proteins in supercontracted wool may be ascribed to the elimination of some of the intermolecular forces such as hydrogen bonds and van der Waals forces by the far more extensive changes in molecular configuration which accompany supercontraction. This may explain the extraction of a greater proportion of the reduced wool at a particular alkaline pH value than from normal wool, fewer cross-links being present to restrain the mutual repulsion of the negatively charged centres in the reduced proteins. Mutual repulsion of negatively charged centres with consequent greater lability of hydrogen bonds was also suggested by Crewther and Dowling (1956) instead of direct splitting of hydrogen bonds (Alexander 1951) to explain supercontraction in LiCl and in some other salt solutions. It was held that if adsorption of Cl^- anions occurs in preference to the highly hydrated Li^+ cations this would increase the net negative charge on the wool proteins thereby favouring structural rearrangements culminating in supercontraction. Reversal of supercontraction by heating to 50°C in alkaline thioglycollate suggests that the disulphide bonds in wool must not be split if the fibre is to remain supercontracted. If they are ruptured the wool proteins revert to their original length.

Increasing the concentration of LiCl beyond 9M may have failed to enhance further the extractability of the proteins by thioglycollate (Fig. 2) through withholding water from them. Water is essential to supercontraction just as it was previously shown to be essential to thermal changes leading to decreased thioglycollate extraction (Lennox 1955). Presumably it facilitates movement of the chemical groups and peptide chains in the wool proteins into new positions.

Non-reversal of supercontraction after heating in an acidic solution of LiBr and subsequently soaking in water was attributed by Alexander (1951) to acid hydrolysis of peptide bonds in the wool proteins. Pretreatment with a boiling solution of 0.12 per cent. H_2SO_4 has also been shown to increase the alkali solubility of wool (Dusenbury, Mercer, and Wakelin 1954). The enhanced extractability with alkaline thioglycollate following supercontraction in 9M LiCl at pH 5, however, arose from the supercontraction itself and not from the accompanying hydrolysis of some peptide bonds due to the low pH of the LiCl solution. This latter possibility is excluded by the observation that heating wool for 20 min at 96°C in buffer at pH 4, or in LiCl solutions of the same pH as the 9M LiCl but of lower concentration, failed to cause either supercontraction or increased extractability in the thioglycollate.

Electron microscopy of the fibrils from wool supercontracted in NaHSO_3 (Jeffrey, Sikorski, and Woods 1955) showed that the protofibrils of the cortical cells, the structures which embody the bulk of the wool protein, themselves undergo

contraction: The enhanced extractability of wool following supercontraction with LiCl is also probably attributable to changes in the extracted fibrillar proteins and not primarily to changes in the membranes or intercellular proteins. This view finds support also in the increased thioglycollate solubility accompanying the supercontraction of the wool cells.

Reversal of the LiCl-enhanced thioglycollate extractability, by heating the supercontracted wool in water (Fig. 5), confirms the fall in extractability after the initial 20 min heating in 9M LiCl (Fig. 4). Supercontraction appears to proceed more rapidly than the hydrothermal changes which lower the extractability or it commences earlier during the heating process. The hydrothermal changes supervene only after the initial 20 min in 9M LiCl.

It is significant that the electrophoretic patterns of the proteins obtained by extraction of the supercontracted wool with thioglycollate are identical in their main features with those obtained from normal wool at higher pH values. This observation suggests that the change in pattern of a thioglycollate extract of normal wool, accompanying an increase in pH of the thioglycollate, for example from 10.5 to 11.0, is not due to alkaline hydrolysis of components appearing in the pH 10.5 extract but reflects an increased extraction of protein components pre-existing in the wool. In this respect it supports earlier observations indicating the absence of hydrolytic damage to the wool proteins under the conditions used in the alkaline thioglycollate fractional extraction method. Thus it has been shown that there was little increase in dialysable nitrogen using five 20-min extractions at 50°C with 0.1M thioglycollate initially at pH 10.5 followed by one extraction with the 0.1M thioglycollate at pH 12.3 (Gillespie and Lennox 1955*a*).

The present authors have also demonstrated that, when 2-ml portions of a 1 per cent. solution of *S*-carboxymethyl keratine 2 (Gillespie 1957) are dialysed through "Cellophane" tubing against 2 l. of 0.1M thioglycollate at pH 10.0, 10.5, 11.0, 11.5, 12.0, or 12.5 for 10 days at 2°C, no protein is lost except at the two highest pH values (12.0 and 12.5) where 5 per cent. of the protein is lost.

Additional evidence of stability was provided by the observation that the amide content of keratine 2 was not less but greater than that of the parent wool (Simmonds 1955; Leach, unpublished data) and that equilibration of wool at pH 13.3 for 24 hr at 25°C did not affect the amide content (Horner 1954). These data also strengthen the view that electrophoresis of the alkaline thioglycollate extracts of wool gives a true picture of the protein components of the fibre, or of groups of components having mobilities corresponding to keratines 1, 2, 3, and 4, and not of the products of alkaline hydrolysis of some unidentified parent protein.

V. ACKNOWLEDGMENT

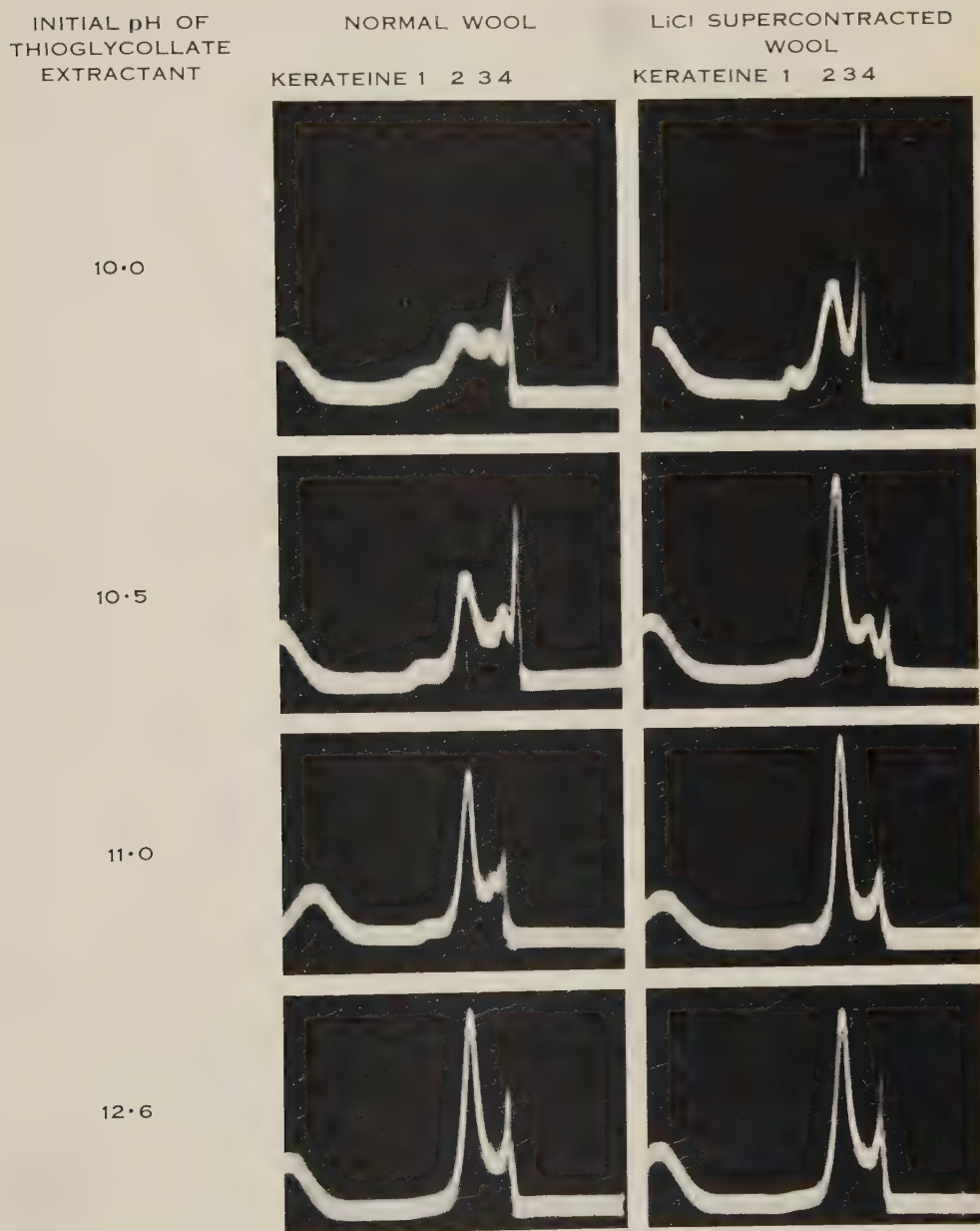
Thanks are due to W. G. Crewther of this Laboratory for supplying the wool cells.

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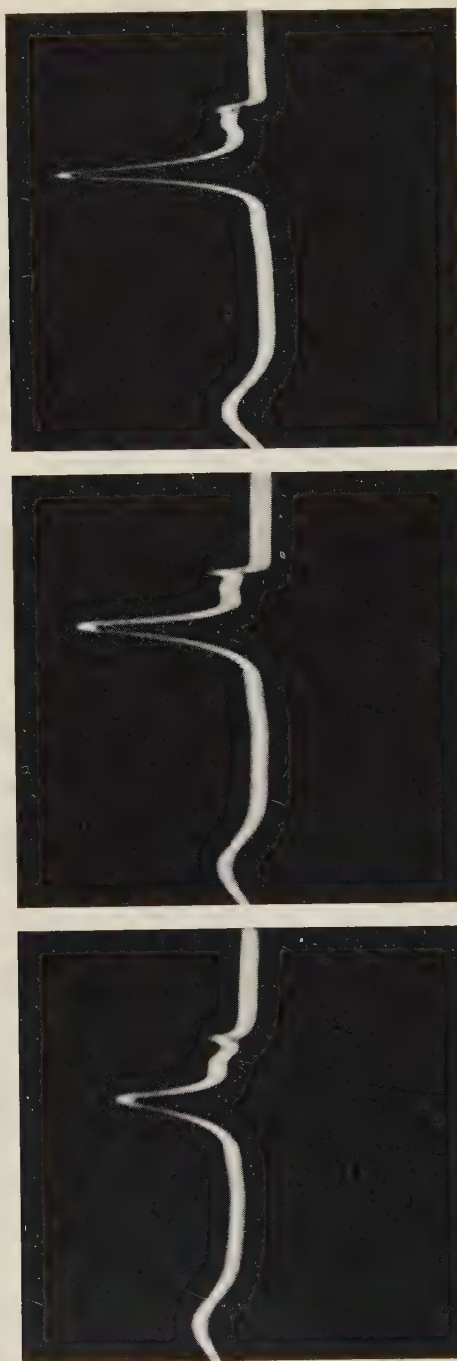
EXTRACTION OF WOOL PROTEINS FOLLOWING SUPERCONTRACTION



Ascending boundary electrophoresis patterns of thioglycollate extracts from normal and 9M LiCl-supercontracted wool run in a thioglycollic acid-glycine-KOH buffer of ionic strength 0.22 at pH 11. Protein concentration 1.5 per cent, except for pH 10.0 extract of normal wool which is at 1.1 per cent. Period of electrophoresis 300 min except for pH 10.5 extract of normal wool (320 min) and pH 11.0 extract of normal wool (250 min).

EXTRACTION OF WOOL PROTEINS FOLLOWING SUPERCONTRACTION

PHENOL SUPERCONTRACTED WOOL



10.5

11.0

12.6

INITIAL pH OF THIOGLYCOLLATE EXTRACTANT

Ascending boundary electrophoresis patterns of thioglycollate extracts of wool supercontracted in 8M phenol. Buffer used was as for Plate 1 and period of electrophoresis was 300 min.

THE FRACTIONATION OF *S*-CARBOXYMETHYL KERATEINE 2 FROM WOOL

By J. M. GILLESPIE*

[*Manuscript received September 25, 1956*]

Summary

The solubility relations of the *S*-carboxymethyl derivative of kerateine 2 (a soluble protein extracted from reduced wool) are studied in systems containing salts in high concentration, mixtures of ethanol and water, and low concentrations of certain divalent cations.

Further purification of this major wool protein is obtained by fractional precipitation with zinc acetate or ammonium sulphate.

I. INTRODUCTION

The preparation and properties of a soluble protein fraction from wool (kerateine 2) were described by Gillespie and Lennox (1953, 1955*a*, 1955*b*). Subsequently the stable *S*-carboxymethyl derivative of kerateine 2 (SCMK2) (Gillespie 1956) was studied in detail by Harrap (1956) and O'Donnell and Woods (1956) using physico-chemical methods. These investigations revealed a great deal of uncertainty concerning the homogeneity of the preparation; electrophoresis, ultracentrifuge, and surface-balance studies indicated that an equilibrium mixture containing the protein in various stages of aggregation was present. The question whether the preparation contained one or more proteins was not settled and the occurrence of aggregation makes the problem extremely difficult to solve. For this reason electrophoresis and ultracentrifuge data are of limited use in separating fractions and in analysing fractions obtained by other means. In the present study, solubility methods as a means of fractionation have been applied to SCMK2 to remove some minor protein constituents. These solubility data will also be of use in investigations dealing with the purification of certain minor components of wool separated in the earlier stage of fractional extraction with alkaline thioglycollate. It will also help to decide whether electrophoretic components appearing in earlier fractions with the same mobility as kerateine 2 are in fact identical with it (cf. Simmonds and Stell 1956). As the purified fraction of SCMK2, which the present investigation makes available, is now also being used in dye uptake, amino acid sequence, end-group, and other similar studies, the method of preparation and properties of this fraction will be described.

II. MATERIALS AND METHODS

(a) Preparation of Kerateine 2 and S-Carboxymethyl Kerateine 2

Full details of the preparation of kerateine 2 were given by Gillespie and Lennox (1953, 1955*a*, 1955*b*). Briefly, solvent-scoured Merino 64's wool top was extracted five times for 20 min at 50°C with 0.1M potassium thioglycollate at an initial pH of 10.5 using 30 ml of extractant per g of wool. The residue was extracted

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once with 0.1M potassium thioglycollate at an initial pH of 12.2* to yield a solution of kerateine 2 containing 0.6–0.7 per cent. protein.

To prepare the *S*-carboxymethyl derivative, the pH of this solution was brought to 9 by the slow addition of acetate buffer, pH 5.0, with stirring and to each litre of solution 40 g of sodium iodoacetate in solution at pH 9 were added. The pH dropped rapidly and was brought to, and maintained at, 8.5 by the dropwise addition of 1N KOH. The nitroprusside test for –SH groups was negative after about 10 min. The solution was dialysed against running tap water for 24 hr, against distilled water for 24 hr, then concentrated by evaporative dialysis to about 2 per cent. protein and stored at –20°C. The original cysteine residues were thereby converted to *S*-carboxymethyl cysteine residues and no measurable amount of cystine remained.

(b) Solubility Curves

The effect of protein precipitants on the solubility of SCMK2 was determined by mixing appropriate amounts of protein solution with precipitant in 40-ml glass-stoppered test tubes to give a final volume of 20 ml; the tubes were then gently rocked at 2°C for 24 hr. In experiments with ethanol a temperature of –5°C was used and the concentrations are recorded as v/v of absolute ethanol. The insoluble protein was centrifuged off at the same temperature and the protein in the supernatant measured spectrophotometrically at 277 m μ . SCMK2 has an extinction coefficient $E_{1\text{ cm}}^{1\%}$ of about 7.6.

(c) Electrophoresis

(i) *Paper Electrophoresis*.—The technique was essentially that of Woods and Gillespie (1953).

(ii) *Moving-boundary Electrophoresis*.—Electrophoresis of the protein fractions was carried out in a Tiselius apparatus at 1°C using a constant potential gradient of about 5.0 V/cm. Prior to electrophoresis the protein solutions were dialysed against 80 vol. of buffer solution for at least 2 days at 2°C.

III. EXPERIMENTAL RESULTS

(a) Solubility Studies by Salting Out

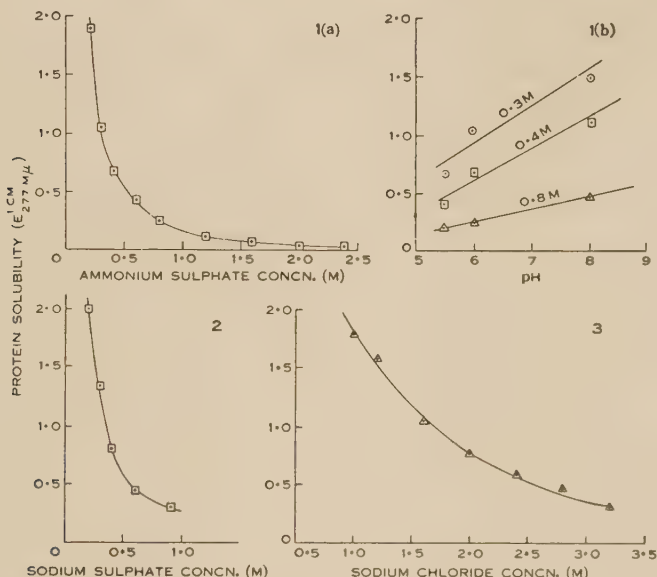
(i) *Ammonium Sulphate*.—A solution of SCMK2 was shaken with ammonium sulphate solution at pH values ranging from 5.5 to 9. The pH was maintained at the selected value by buffers of ionic strength 0.1. Typical precipitation curves are shown in Figures 1(a) and 1(b). The protein becomes progressively less soluble as the pH is lowered, most of the protein being very easily salted out, but one fraction requires a considerably higher concentration of ammonium sulphate for precipitation.

(ii) *Sodium Sulphate*.—A solution of SCMK2 was shaken with sodium sulphate solution in a similar manner to that followed using ammonium sulphate. The solubility results at pH 6.0 (Fig. 2) are almost identical with those for ammonium sulphate up to the solubility limit of sodium sulphate.

* Originally pH 12.3 was recommended but pH 12.2 gives an electrophoretically purer product.

(iii) *Sodium Chloride*.—SCMK2 can be salted out even by sodium chloride. The results obtained at pH 6 are shown in Figure 3.

(iv) *Salting-out Data*.—The data from certain of these experiments have been combined in Figure 4, where the ionic strength has been plotted against log solubility



Figs. 1-3.—Solubility of SCM2 at pH 6 as a function of ammonium sulphate concentration (Fig. 1(a)); as a function of pH at various ammonium sulphate concentrations (Fig. 1(b)); and at pH 6 as a function of sodium sulphate (Fig. 2) and sodium chloride (Fig. 3) concentrations.

* (Cohn and Edsall 1943). With these three precipitants a linear plot is obtained to which can be fitted the equation

$$\log S = \beta' - K_s'(\Gamma/2),$$

where β' = the extrapolated intercept of the linear portion of the curve on the ordinate axis, and

$\Gamma/2$ = the ionic strength expressed as g/1000 g of water.

The salting-out constant K_s' can be obtained from the slope of such curves and the values are characteristic of a particular precipitant and a particular protein. The values obtained in these experiments were: ammonium sulphate 1.43, sodium sulphate 1.20, and sodium chloride 0.32. The protein used for these experiments was an ammonium sulphate-precipitated fraction prepared as described in Section IV(b). It can be seen from Figure 4 that this protein appears to be salted out slightly more easily by ammonium sulphate than by sodium sulphate; this is the reverse of the usual order.

(b) *Precipitation with Ethanol*

Figure 5 shows some typical solubility curves obtained with ethanol as the precipitant of SCMK2. It can be seen that increasing the ionic strength from 0.0001

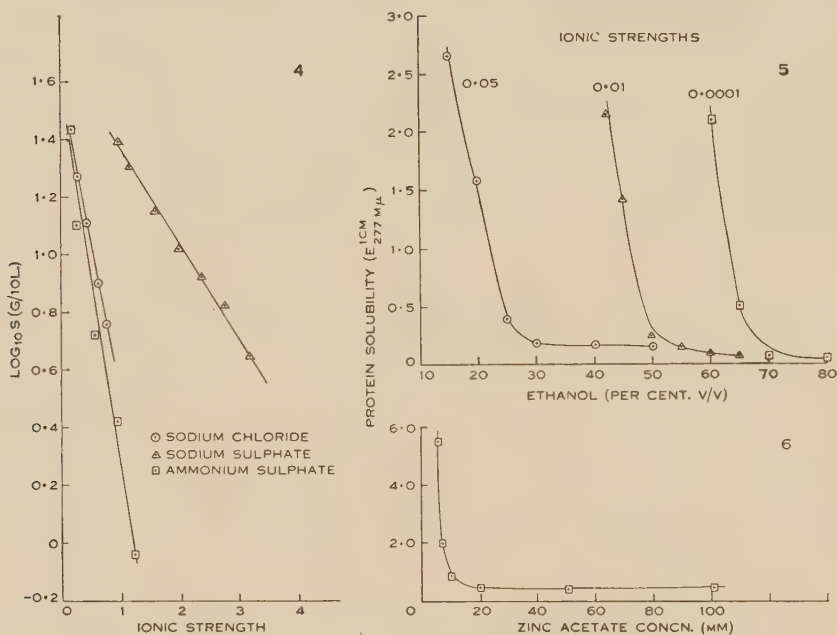


Fig. 4.—Solubility of SCMK2 fraction P₂ as a function of ionic strength.

Fig. 5.—Solubility of SCMK2 at -5°C as a function of ethanol concentration at various ionic strengths.

Fig. 6.—Solubility of SCMK2 as a function of zinc acetate concentration at pH 6.

to 0.05 greatly decreased the protein solubility. This is the reverse of the usual behaviour. Moreover, the protein precipitate, unlike most ethanol-protein precipi-

TABLE 1

EFFECT OF TEMPERATURE ON THE SOLUBILITY OF S-CARBOXYMETHYL KERATEINE 2 IN ETHANOL-WATER MIXTURE

System at pH 6.0 in acetate buffer of ionic strength 0.002

Ethanol (%)	Protein Solubility ($E_{277}^{1\text{cm}}$)	
	-5°C	-20°C
50	1.8	—
60	0.3	2.2
70	0.08	0.25
80	0.03	0.16

tates, was translucent and tended to extend throughout the liquid. After centrifugation, its volume was still considerable and further amounts of supernatant could

be expressed by compression. The protein became less soluble as the pH was reduced from 8 towards 5. As the operating temperature was reduced from -5 to -20°C at an ionic strength of 0.002 the protein became more soluble (Table 1).

(c) *Precipitation with Divalent Metal Acetates*

The precipitating effect of acetates of divalent cations at 0.02M concentration at pH 6.0 in acetate buffer of ionic strength 0.01 after holding for 18 hr at 2°C is shown in Table 2. Copper salts absorb ultraviolet light strongly and so the protein concentration of the supernatants from this test could not be measured spectrophotometrically. Manganese seemed worthy of further study but at pH 6.0, which was selected as the most convenient pH value for these tests, the supernatants rapidly became turbid and so no further work was done with this cation. Although salts of

TABLE 2
SOLUBILITY OF S-CARBOXYMETHYL KERATEINE 2 IN THE PRESENCE OF
CERTAIN DIVALENT CATIONS

All the ions were tested at 20 mM concentration except in one of the tests with calcium. System at pH 6.0 in acetate buffer of ionic strength 0.01

Cations	Protein Solubility ($E_{277}^{1\text{ cm}}$)	Cations	Protein Solubility ($E_{277}^{1\text{ cm}}$)
Mg ⁺⁺	3.40	Cd ⁺⁺	0.075
Ca ⁺⁺	1.77	Pb ⁺⁺	0.055
Ca ⁺⁺ (50 mM)	0.84	Hg ⁺⁺	0.005
Mn ⁺⁺	0.65	Cu ⁺⁺	Heavy precipitate
Zn ⁺⁺	0.28		

zinc do not precipitate SCMK2 as completely as do the salts of some other divalent cations, this was judged to be an advantage since it could lead to a method of further fractionation. For this reason, and since zinc salts have proved particularly useful in studies with other proteins (e.g. Cohn *et al.* 1950), they were selected for further investigation.

(d) *Precipitation with Zinc Acetate*

(i) *Effect of Concentration.*—The effect of the concentration of zinc acetate on the solubility of SCMK2 in acetate buffer of ionic strength 0.01 at pH 6 with an initial protein concentration of about 0.8 per cent. is shown in Figure 6. It can be seen that the protein was almost completely precipitated by 10 mM zinc acetate but that there was a residue of protein accounting for about 10 per cent. of the optical density which was not precipitated even by 100 mM zinc acetate. In later experiments with purified fractions it was found that the concentration of zinc acetate for complete precipitation was somewhat lower, being of the order of 7 mM, and, within the limits of experimental error, this did not vary as the protein concentration increased from 0.05 to 1.0 per cent.

(ii) *Effect of pH.*—The effect of pH on the precipitation of SCMK2 with zinc acetate was studied over the pH range 5.5–9 in tris(hydroxymethyl)amino-

methane(TRIS)-acetate buffer of ionic strength 0.02 containing about 0.5 per cent. protein and 10 mM zinc acetate; the mixture was equilibrated at 2°C for 24 hr. It was found that the solubility decreased as the pH was increased to 5.8. It remained at this low level up to about pH 7.5 and increased sharply at higher pH values.

(iii) *Effect of Ionic Strength.*—Figure 7 shows the effect of ionic strength on the solubility of SCMK2 in the presence of 20 mM zinc acetate at pH 6. Acetate buffer of ionic strength 0.01 was used and the ionic strength was varied by the addition of sodium chloride. It can be seen that the amount of protein in the supernatant

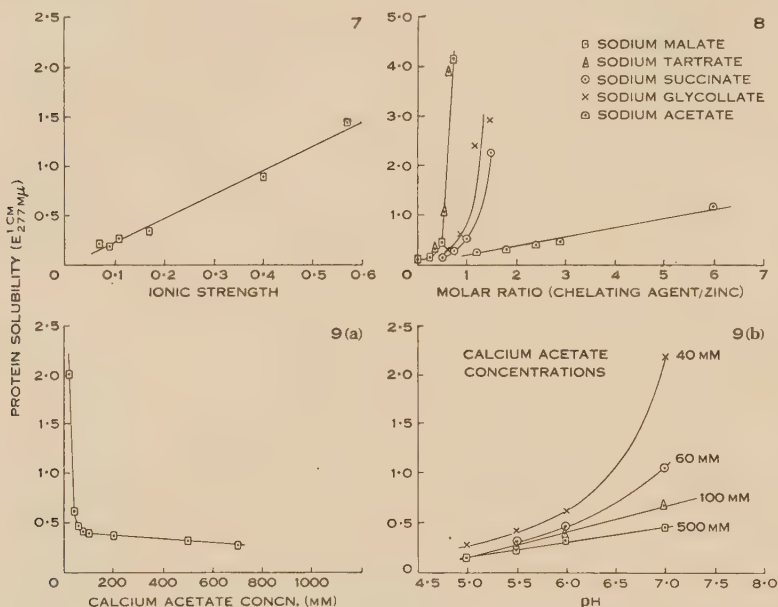


Fig. 7.—Solubility of SCMK2 in 20 mM zinc acetate at pH 6 as a function of ionic strength.

Fig. 8.—Effect of chelating agents on the solubility of SCMK2 in the presence of 20 mM zinc acetate.

Fig. 9.—Solubility of SCMK2 (a) at pH 6 as a function of calcium acetate concentration; and (b) as a function of pH at various calcium acetate concentrations.

increased as the ionic strength was increased. It is not known whether this was due to salting in or to removal of bound zinc ions from the protein.

(iv) *Effect of Temperature.*—The zinc-protein complex was found to be less soluble at 20°C than at 2°C.

(v) *Fractional Extraction of the Zinc Precipitate with Chelating Agents.*—Isliker and Antoniadis (1955) separated antibodies from γ -globulin by selectively dissociating zinc-protein complexes with chelating agents which compete with the protein for the cation. This technique has been applied to the insoluble zinc-protein complex of SCMK2. The procedure followed was similar to that employed in the solubility

experiments except that the chelating agent was added in concentrations up to 250 mM. The other variables were kept constant: the zinc concentration at 20 mM, the pH at 6, and protein concentration at 0.5 per cent; each tube contained acetate buffer of ionic strength 0.01. The tubes were equilibrated overnight at 2°C with gentle rocking. Four chelating agents* were tested for their ability to fractionally extract the zinc-protein complex and for comparison acetic acid was also included. Figure 8 shows that under the conditions used there was no evidence of fractionation. It was apparent also that the chelating agents competed much more strongly for the zinc than did the protein. No suitable material forming complexes with zinc ions with association constants slightly greater than the zinc-protein complex constant has yet been found.

TABLE 3

SOLUBILITY OF S-CARBOXYMETHYL KERATEINE 2 IN ETHANOL-WATER MIXTURES
WITH AND WITHOUT THE ADDITION OF ZINC ACETATE

System contained 0.7 per cent. protein in acetate buffer of ionic strength
0.01 at pH 6.0

Ethanol Concentration (%)	Protein Solubility ($E_{277}^{1\text{ cm}}$)	
	No Zinc	0.002M Zinc
20	>5	1.6
25	>5	0.4
30	>5	0.12
42	2.15	—
45	1.45	—
50	0.25	—

(vi) *Precipitation with Ethanol in the Presence of Zinc Acetate.*—Small concentrations of zinc acetate, too low to precipitate SCMK2 from water, reduced its solubility in ethanol-water mixtures to a marked degree (Table 3).

(e) *Precipitation with Calcium Acetate*

Calcium salts resemble zinc salts in precipitating part of the protein of SCMK2 but, as Figures 9(a) and 9(b) show, for precipitation to occur to any extent the concentration of calcium acetate required is five to 10 times greater than the concentration of zinc acetate. These experiments were carried out in a TRIS-acetate buffer of ionic strength 0.01 at an initial protein concentration of about 0.5 per cent. The pH of minimum solubility was lower than that observed in the presence of zinc acetate and the solubility steadily decreased as the pH was lowered towards 5, the lowest pH examined.

* Malic, tartaric, succinic, and glycollic acids.

IV. PREPARATION OF FRACTIONS OF *S*-CARBOXYMETHYL KERATEINE 2*(a) Precipitation with Zinc Acetate*

SCMK2 was fractionally precipitated from a solution of ionic strength 0.01 and at pH 6 containing 1 per cent. protein and 20 mM zinc acetate at 2°C. The precipitate was centrifuged off at 2°C, twice suspended in zinc acetate-sodium acetate solution of the same pH and concentration, and recentrifuged. Both precipitate and supernatant were treated with excess ethylenediaminetetra-acetate or citrate at pH 9 and then dialysed thoroughly to remove the zinc. The SCMK2 zinc-precipitated fraction (P_1), usually representing about 90 per cent. of the protein, gave slightly turbid solutions whilst the SCMK2 zinc supernatant fraction (S_1) gave very clear solutions and dissolved easily even after freeze-drying, in contrast to the lower solubility of both the unfractionated SCMK2 and P_1 under these conditions. Electrophoretically also, P_1 showed most of the features of the original protein, appearing fairly homogeneous in dilute solution and giving evidence of aggregation at high concentrations, but S_1 gave evidence of considerable heterogeneity. Typical electrophoresis patterns obtained at pH 6 in acetate buffers of ionic strength 0.1 are shown in Plate 1. P_1 became insoluble at pH values below 4.5 whilst S_1 remained in solution over the entire pH range. It also required a much higher concentration of ammonium sulphate for complete precipitation than did P_1 . S_1 could be precipitated by mercury and lead salts but not by zinc, calcium, or manganese acetates.

Analysis of SCMK2 and of these two fractions showed that they differ in the proportions of the constituent amino acids (Simmonds, unpublished data). Optical density measurements at 278 $m\mu$ have shown that S_1 contains about two-thirds as much aromatic acids as compared with P_1 . Surface balance molecular weight determinations of the two fractions are given in Appendix I.

(b) Salting Out with Ammonium Sulphate

The solubility curves described earlier suggest that at pH 6 one component of SCMK2 is completely precipitated by 0.4M ammonium sulphate. A large-scale preparative fractionation was made by dialysing the required amount of ammonium sulphate into a 0.5 per cent. solution of SCMK2 at 2°C containing 0.1M acetate buffer at pH 6. The precipitate was centrifuged off and washed once with 0.4M ammonium sulphate containing 0.1M acetate at pH 6. The protein was then dissolved in a lesser volume of water containing NaHCO_3 at pH 9, thoroughly dialysed, and reprecipitated and treated as before.

This SCMK2 ammonium sulphate-precipitated fraction (P_2) was salted out over a very narrow range of ammonium sulphate concentration as shown in Figure 10. The same figure shows that the ammonium sulphate supernatant fraction (S_2) precipitated over a very wide range of concentration. P_2 was shown to be completely precipitable with zinc acetate and represents about 85 per cent. of the original protein. S_2 is partly precipitable with zinc acetate (about 45 per cent.) and this may be due to incomplete precipitation with ammonium sulphate. P_2 appears to be identical with P_1 . Experiments have been conducted in the Tiselius electrophoresis apparatus with P_2 at pH 6, and typical patterns obtained at this pH in acetate buffers of ionic

strength 0.1 at a protein concentration of 1 per cent. are shown in Plate 1. The ultraviolet absorption curve of P_2 (Fig. 11) is a typical protein curve, and the extinction coefficient for a 1 per cent. solution at $278\text{ m}\mu$ was found to be about 8.

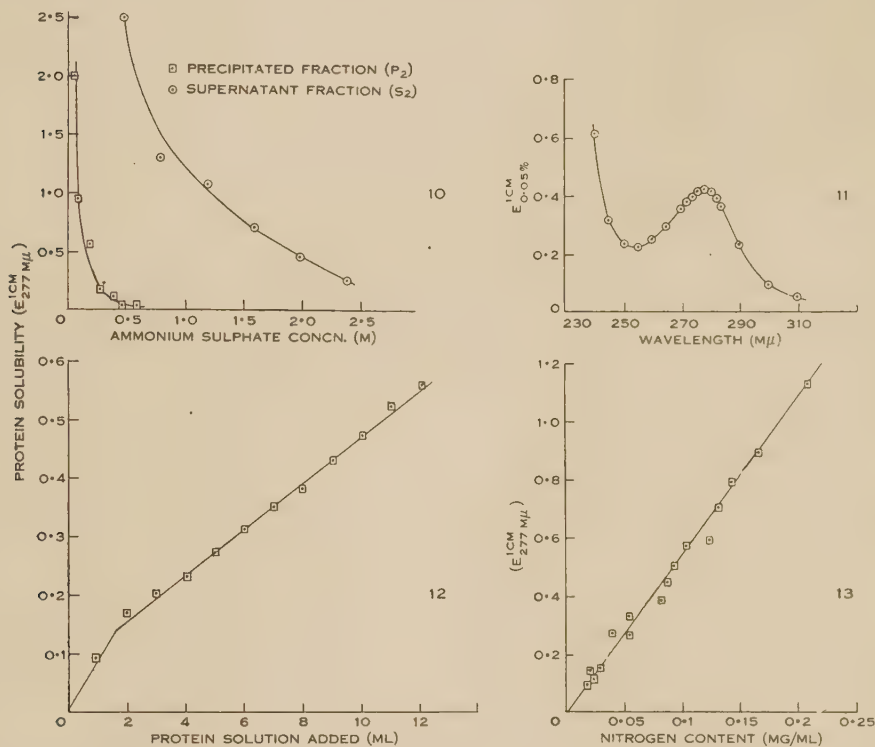


Fig. 10.—Solubility of SCM2 fractions P_2 and S_2 as a function of ammonium sulphate concentration at pH 6.

Fig. 11.—Ultraviolet absorption spectrum of SCM2 fraction P_2 at pH 7.

Fig. 12.—Solubility of SCM2 fraction P_2 in 0.1M sodium sulphate at pH 6 as a function of protein concentration.

Fig. 13.—Specific property solubility test of SCM2 fraction P_2 .

(c) Tests for Homogeneity of SCM2 Fractions

Most of the experiments to be described have been carried out on an ammonium sulphate-precipitated fraction but the results apply equally well to a zinc-precipitated fraction. As many physicochemical tests for homogeneity are of reduced usefulness in an aggregating system, it was decided to obtain as much information as possible from solubility studies. Falconer and Taylor (1946) list the following three types of solubility test: (1) the variable solvent solubility test, (2) the constant solvent solubility test, and (3) specific property test. Test (1) was used throughout the earlier part of this work but showed no breaks in the curve indicative of different components. The smooth curves shown in Figure 10 are typical of those obtained in numerous experiments, some of which were based on much smaller increments in salt concentration. Test (2) was studied from both the supersaturated and the unsaturated side.

The former experiments were carried out by measuring the protein solubility in a series of tubes containing variable amounts of protein solution but with all other conditions constant. Figure 12 shows that, unlike a system containing a single protein, increasing the total protein in the system also increased the protein in solution over the entire range of protein additions. Experiments on the unsaturated side are more normal but should give the same result. However, the technique was very difficult with P_2 because the protein precipitate forms gel aggregates and it cannot be pipetted as a uniform suspension; nor can a uniform sample be withdrawn from the test mixture for a determination of total protein in the system. For this reason experiments on the unsaturated side had to be abandoned. Test (3), the specific property solubility test, has much to recommend it, for unlike the others, aggregation cannot affect the results. It proved to be of the greatest utility in dealing with enzymes (Falconer and Taylor 1946; Pfaff and Demain 1956) where the specific activity differentiates clearly between enzymic and non-enzymic proteins. The method can be applied to wool proteins using estimates of certain amino acids but the analytical methods are less sensitive than those used for estimating enzyme activity and lower sensitivity must therefore be expected.

When the absorption at $278\text{ m}\mu$ was plotted against the nitrogen content of a series of supernatants obtained by partial precipitation of P_2 with sodium sulphate, there were no obvious deviations from a straight line (Fig. 13).

(d) Separation by Paper Electrophoresis

Electrophoresis of SCMK2 on paper (Whatman 3MM) was unsatisfactory at all pH values between 6 and 11 due to adsorption on the paper and resultant streaking similar to that observed with papain (Woods and Gillespie 1953). The addition of 8M urea to the buffer did not prevent adsorption.

V. DISCUSSION

Harrap (1956) and O'Donnell and Woods (1956) established that keratine 2 and its derivative, SCMK2, are aggregating systems. In the light of this, some of the anomalous solubility data can be interpreted. Thus it could explain why the solubility in ethanol-water mixtures decreases with increasing salt concentrations, which is the converse of usual experience, although Cohn *et al.* (1946) published a schematic solubility curve which indicates that some globulins are salted out by an increase in salt concentration on one side of the isoelectric point whilst showing the reverse behaviour on the other side. However, the wool protein at the lowest ionic strengths will be partly disaggregated and therefore rather soluble in ethanol whilst at the higher salt concentrations it will be more aggregated and therefore more easily precipitated by ethanol. The observation that an increase in temperature decreases the solubility of SCMK2 in ethanol-water mixtures and the solubility of the zinc acetate precipitate in water may also be due to increased aggregation at the higher temperatures. This hypothesis has not been subjected to experimental verification, but the electrophoretic patterns of these proteins are profoundly changed by increasing the operating temperature from near 1°C to 20°C . Other proteins have a negative temperature coefficient of solubility, e.g. horse serum albumin (McMeekin

1939), insulin sulphate, the Bence Jones protein (Cohn and Edsall 1943), and an elastin extract (Partridge, Davis, and Adair 1955); it is common also with many proteins in concentrated salt solutions (Cohn and Edsall 1943). SCMK2 is very easily salted out, even univalent anions sufficing, and in this respect the protein resembles fibrinogen. This behaviour again may be a reflection of increasing aggregation with increasing salt concentration.

There are certain features of the binding of zinc by the protein that are of great interest. The complex is very insoluble in the presence of excess zinc but rapidly dissolves when washed with water. The association constant must therefore be rather low. The binding is being further studied in this Laboratory using radioactive zinc as a tracer in dialysis equilibrium experiments (Gillespie and Springell, unpublished data). The pH range of 6–7 in which the zinc-protein complex is least soluble is similar to that observed for γ -globulin (Islaker and Antoniadis 1955) and for human plasma albumin (Cohn *et al.* 1950). The SCMK2 calcium-precipitated fraction, on the other hand, has a lower pH of minimum solubility. The exact value could not be determined because of the insolubility of SCMK2 below pH 5, but it apparently lies in the vicinity of the value 5 reported by Cohn *et al.* (1950) for the calcium precipitation of certain plasma proteins. The evidence clearly indicates that about 90 per cent. of the protein in SCMK2 differs from the rest and can be separated either by fractional precipitation with high concentrations of salts or with much lower concentrations of zinc acetate. In each case the minor protein is the more soluble fraction. It is also electrophoretically heterogeneous and may represent some of the minor constituents of wool not removed during earlier stages in the preparative fractional extraction.

The fractionated protein could not be sub-fractionated by any of the means employed. The absence of any points of inflection on the curves from the variable solvent solubility test suggests that it is either one protein or a group of closely related proteins. The evidence from the specific property solubility test is not completely convincing due to experimental difficulties but it suggests no significant amount of impurity. The marked deviation of the curve from the horizontal in the constant solvent solubility test suggests that the material is not homogeneous. However, it is not certain how far this test and the variable solvent test can be relied upon in an aggregating system. It is known that variation in such factors as salt concentration and protein concentration cause considerable changes in the degree of aggregation of wool proteins (O'Donnell and Woods 1956) and it is possible that these factors may upset the tests. However, fractions P_1 and P_2 represent the purest wool protein preparations yet available. The broad problem still remains as to whether this precipitable material is a single protein or not, and whether it is possible to decide unequivocally with an aggregating system whether only one protein is present or many.

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APPENDIX 1

MOLECULAR WEIGHTS OF S-CARBOXYMETHYL KERATEINE 2 FRACTIONS

By B. S. HARRAP*

The molecular weights of the fractions of *S*-carboxymethyl kerateine 2 have been determined by the surface balance technique described by Harrap (1955a, 1955b, 1956). Each of the four fractions was spread on subsolutions of ionic strength

TABLE 4

MOLECULAR WEIGHTS OF FOUR S-CARBOXYMETHYL KERATEINE 2 FRACTIONS SPREAD ON BUFFERS OF IONIC STRENGTH 0.01

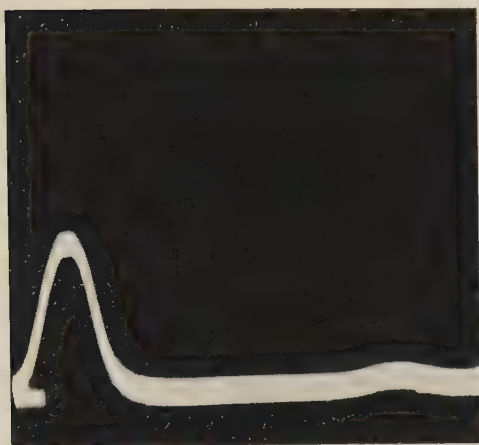
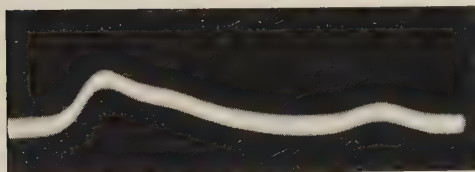
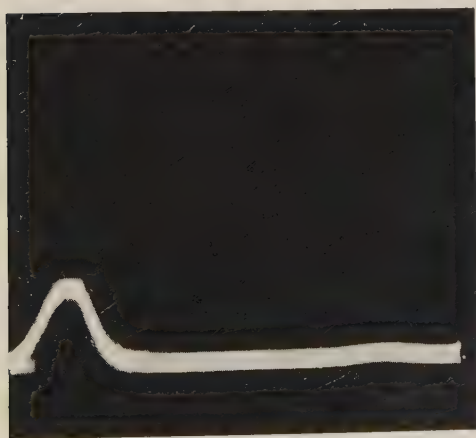
Fraction	pH		
	2.0	6.9	9.0
Zinc supernatant (S ₁)	16,000	11,500	9,000
Ammonium sulphate supernatant (S ₂)	19,500	12,000	8,500
Zinc precipitated (P ₁)	27,000	22,000	10,500
Ammonium sulphate precipitated (P ₂)	12,500	10,500	9,500

0.01 at pH values of 2.0, 6.9, and 9.0; the buffers used being hydrochloric acid, phosphate, and borate respectively. The results, which are accurate to about ± 5 per cent., are shown in Table 4.

It is clear that all four fractions may undergo aggregation or disaggregation as the pH of the system is varied, but at pH 9.0 each shows its least molecular weight over the pH range studied. Moreover, there is little difference between the molecular weights of the fractions at this pH, although over a series of replicate experiments the values for the two precipitated fractions were consistently higher than those for

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FRACTIONATION OF WOOL PROTEINS

DESCENDING BOUNDARY
←ASCENDING BOUNDARY
→ZINC-PRECIPIATED FRACTION (P_1) : 1% PROTEIN; 300 MINZINC SUPERNATANT FRACTION (S_1) : 0.65% PROTEIN; 250 MINAMMONIUM SULPHATE-PRECIPIATED FRACTION (P_2) : 1% PROTEIN; 300 MIN

Electrophoresis of SCMK2 fractions run in acetate buffer at pH 6 and ionic strength 0.1 at 5.0 V/cm.



the supernatant fractions. The similarity in molecular weight suggests that this factor cannot be responsible for the fractionation obtained on the basis of solubility.

At pH 6.9 there seems to be rather more aggregation in the case of the supernatant fractions (S_1 and S_2) as compared with the ammonium sulphate-precipitated fraction (P_2). This suggests that the reduction in net charge on lowering the pH is greater for the former two fractions as compared with the last and points to differences in their amino acid compositions. More interesting is the difference between the molecular weights of the two precipitated fractions, the value for the zinc fraction (P_1) being about twice that for the ammonium sulphate fraction (P_2). This raises the possibility that part of the zinc remains firmly bound to the protein even after the treatment with a chelating agent and prolonged dialysis, causing dimerization of the protein derivative.

At pH 2.0 the supernatant fractions again show more aggregation than the ammonium sulphate-precipitated fraction. So far as their molecular weights are concerned, this greater tendency to aggregate as the pH is reduced seems to be the main difference between the precipitable and non-precipitable fractions. The difference in aggregation tendency may account for the comparative homogeneity of the unfractionated *S*-carboxymethyl keratine 2 towards electrophoresis and ultracentrifugation under some conditions and its heterogeneity under others (Gillespie 1956; O'Donnell and Woods 1956).

The large difference between the molecular weights of the zinc- and ammonium sulphate-precipitated fractions is still evident at pH 2.0. This suggests that if zinc binding is the cause, the zinc cannot be bound to the carboxyl side-chains on the protein since they would be unionized at this pH.

ORTHO-PARA CORTICAL DIFFERENTIATION IN "ANOMALOUS" MERINO WOOL

By NOOR AHMAD* and W. R. LANG*

[*Manuscript received July 13, 1956*]

Summary

"Anomalous" or "doggy" Merino wool appears to be an increasing problem in Australian woolgrowing. The pattern of the ortho-para cortical differentiation of the straighter-fibred anomalous growth has been compared with that of normal wool. It was found that demarcations of ortho- and paracortex typical of much coarser fibres, may be present in the fine fibres of anomalous growth. In general, doggy wool is coarser than the normal flock mean and has a higher percentage occurrence of paracortex in the cross section.

I. INTRODUCTION

Bilateral cortical effects have been recognized by a number of workers (McMurtrie 1886; von Bergen 1935; Hirabayasha 1938; O'Hara 1938) but Horio and Kondo (1953) recognized that there are two cortical components in the form of approximate hemi-cylinders wound round each other helically in phase with the fibre crimp. The two components differed in dyeing, swelling in alkali, and in birefringence after exposure to alkali. Fraser and Rogers (1953, 1954) showed that the outer bilateral component of the crimp wave had an affinity for basic dyes and was less resistant to thioglycollate digestion. Fraser, Lindley, and Rogers (1954) attributed the cause of the appearance in segmentation to the difference in the amino acid composition, in cystine, proline, and dicarboxylic acids. Other evidence of a difference in physical and chemical properties has been given by Race (1946), Woods (1953), Dusenbury, Mercer, and Wakelin (1954), Mercer, Golden, and Jeffries (1954), and Golden, Whitwell, and Mercer (1955). The basophilic component has been called orthocortex or S (soft) and the other, the paracortex or H (hard).

Fraser and Rogers (1953) noted that in wool of 64's quality wherein the cortex was differentiated on the basis of basophilic dyeing, there was no gradation of properties near the boundary. The paracortex was the lesser in area and the segmental line lay along the major axis of the fibre cross section. From examination of coarser wool, Fraser and Rogers (1955) made the following suggestions:

- (i) In wool less than $25\ \mu$ in diameter, the segments are discrete.
- (ii) Between 25 – $35\ \mu$, the demarcation is less sharp and the uptake of dye variable. Frequently ortho- surrounded by paracortex and vice versa are evident.
- (iii) Above $35\ \mu$, the division between ortho- and paracortex is no longer justifiable, on the basis of staining methods. There is a marked tendency for the paracortex to become concentrated around the periphery in the coarser fibres, leaving a central core of orthocortex.

The disappearance of segmentation appears to be a function of fibre diameter.

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A Merino woolgrowing problem of some standing in Australia is that typically well-crimped fleeces may show a proportion of "anomalous", "doggy", or "rough" Merino fleeces, in which the crimp has a longer wavelength than would have been expected and a shiny staple appearance, causing the adoption of the colloquial description "doggy". When a number of these samples, chosen by experts in several Australian States, was received for study, one aspect examined was the cortical response to basic dyeing. The results were unexpected enough to report separately.

II. MATERIALS AND METHODS

(a) Dyeing Technique

After degreasing in petroleum ether and warm distilled water, the samples were dehydrated in ethyl alcohol and then air-dried. Fine wools were dyed with 0.08 per cent. methylene blue, but, for adequate dyeing, the doggy wool required 0.08–0.12 per cent. Dyeing was carried out in 0.03M potassium dihydrogen phosphate buffer of pH 7.4 at 100°C for 30 min with a wool/liquor ratio of 0.1 g per 30 ml.

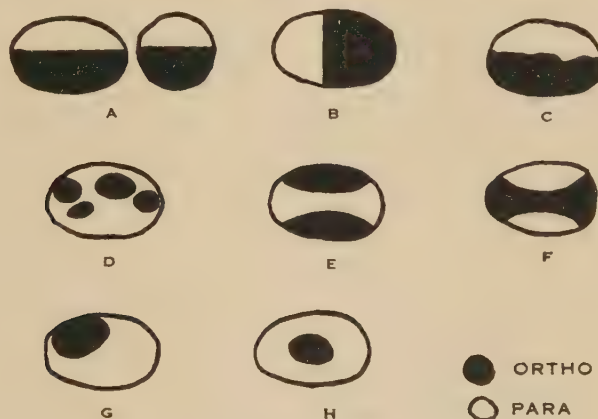


Fig. 1—An arbitrary classification of types of bilateral cortical segmentation.

III. RESULTS

(a) Types of Segmentation

It was necessary to establish an arbitrary classification for the various types of segmentation. This was made as follows:

- | <i>Type</i> | <i>Description</i> (see Fig. 1) |
|-------------|---|
| <i>A</i> | Clear demarcation, approximately along the major axis of the elliptical section. If fibre circular, then included in <i>A</i> if demarcation clearly defined. |
| <i>B</i> | Clear demarcation approximately along the minor axis of the elliptical section. |
| <i>C</i> | Demarcation not well defined, due to a gradation in staining. |
| <i>D</i> | When the orthocortex was gathered in two or more separated groups, usually being of smaller total area than the paracortex. This was of rare occurrence. |

TABLE 1
NORMAL MERINO WOOL: MEASUREMENTS ON SECTIONS FROM THREE ADJACENT STAPLES FROM THE SAME FLEECE

Staple	Section	Para-cortex (%) [*]	S.D.	Mean Thickness (μ)	Types of Segmentation		Mean Thickness of B (μ)
					A (%)	B (%)	
I	1	39.6	9.2	20.6	86	14	19.8
	2	40.7	7.9	20.6	86	14	20.6
	3	39.6	7.4	20.8	93	7	18.0
	4	37.7	9.1	21.1	84	16	19.6
II	1	41.8	7.7	21.6	92	8	20.3
	2	40.7	8.0	22.1	88	12	21.4
	3	37.0	7.8	21.5	90	10	21.2
	4	37.4	8.0	22.3	84	16	20.8
	5	40.0	7.2	21.9	89	11	19.0
	6	40.4	7.0	21.6	89	11	19.3
III	1	41.9	6.9	22.6	93	7	22.0
	2	37.2	6.4	22.5	92	8	21.6
	3	41.6	7.6	23.5	94	6	22.7
	4	40.4	8.2	21.8	83	17	21.5
	5	41.1	8.8	21.8	84	16	21.5
	6	44.5	8.2	22.4	94	6	17.6

^{*} Mean = 40.1 per cent. Standard error of mean = 1.96 per cent.

TABLE 2
PERCENTAGE TYPES OF SEGMENTATION IN NORMAL MERINO WOOL
Values are arranged in order of increasing mean thickness

Sample No.	Para-cortex (%) [*]	S.D.	Mean Thickness (μ)	Types of Segmentation (%) [†]						
				A	B	C	D	E	F	G
N3	35.5	5.9	15.8	76	24					
N2	38.1	5.4	18.1	76	24					
N5	35.5	6.2	19.2	80	20					
N1	42.7	5.9	19.6	85	15					
N11	37.3	8.5	19.6	64	36					
N4	31.3	5.8	22.0	80	20					
N9	31.3	5.1	22.3	74	24	2 (33)				
N12	36.4	7.7	22.3	60	38					2 (17)
N13	37.8	6.8	22.3	66	34					
N14	40.1	5.5	22.7	72	28					
N10	38.2	6.3	22.8	66	24	2 (26)			8 (27)	
N7	37.2	6.3	23.9	90	10					
N8	36.5	8.4	27.5	84	14					
N6	45.3	9.2	35.9	18	2	68 (36)	4 (41)	8 (46)		

^{*} Mean = 37.3 per cent.

[†] Mean thickness of the group is given in parentheses.

- E* Resembling *D*, but with two separate occurrences of orthocortex situated peripherally and diametrically opposed, leaving a channel of paracortex centrally across the fibre.
- F* *E* with ortho- and paracortex reversed in position.
- G* A single, small, circular occurrence of orthocortex at the fibre periphery.
- H* A single circular occurrence of orthocortex at the fibre centre.

TABLE 3

PERCENTAGE PARA-CORTEX AND CLASSIFICATION OF TYPES OF SEGMENTATION IN ANOMALOUS MERINO WOOL SAMPLES

Sample size = 50 fibres. Values are arranged in order of increasing mean thickness

Sample No.	Para-cortex (%)*	S.D.	Mean Thickness (μ)	Types of Segmentation (%)							
				<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	<i>F</i>	<i>G</i>	<i>H</i>
D2	74	9.9	21.8	—	—	—	—	—	—	—	100
D19	59	12.0	23.0	8	—	—	—	—	—	24	68
D30	35	9.2	23.4	36	32	20	—	—	4	6	—
D28	47	8.9	24.1	46	18	12	—	—	14	8	—
D10	51	14.8	24.4	26	—	24	4	—	4	22	18
D17	47	12.5	25.8	42	—	30	—	—	4	20	4
D25	56	14.3	26.4	24	—	10	4	—	—	4	58
D20	47	13.9	26.5	16	4	40	8	2	10	20	—
D11	69	18.5	26.6	24	—	10	4	—	—	4	58
D1	85	11.9	26.7	—	—	—	—	—	—	—	100
D29	38	13.3	26.8	30	6	48	—	—	14	2	—
D15	50	11.3	27.0	40	—	28	—	2	2	10	14
D4	63	14.7	27.8	2	2	10	—	—	2	26	56
D6	60	15.6	28.0	16	—	40	2	—	—	26	14
D7	63	10.3	28.3	12	—	26	12	4	8	32	6
D9	61	11.8	28.6	8	—	16	—	—	—	24	52
D23	50	13.3	29.0	14	2	34	—	—	8	32	10
D31	46	10.2	29.0	50	10	12	2	4	10	10	2
D8	56	9.1	29.6	12	—	28	—	—	2	40	18
D14	56	17.0	30.6	16	—	32	6	—	—	14	26
D12	45	12.5	33.3	32	—	14	—	—	14	18	2
D5	53	12.5	34.2	—	—	34	26	—	—	30	10
D18	60	10.0	34.3	—	—	2	—	—	—	4	88
D3	45	11.1	34.7	8	—	22	—	4	46	14	2

* Mean = 55 per cent.

(b) Dimensional and Bilateral Cortical Phenomena in Doggy Wool

Seventeen normal wool samples were added to the group of 31 doggy or rough Merino wools, so that comparisons could be made more easily. As a guide to the repeatability of measurements on the respective areas of ortho- and paracortex in cross sections from the same staple and from adjacent staples in the same fleece, three such staples were sectioned at a number of levels after staining. Table 1 shows that the percentage of paracortex was fairly constant. Areas were assessed by copying the microprojections at 500 diameters on to millimetre graph paper, from whence the estimates were made.

It is apparent that the percentage by area of paracortex is reasonably constant in the three staples that the percentage of the cross sections in which the segmentation follows the major axis lies between 83 and 94 per cent., that the mean thickness of fibre sections with the segmentation following the minor axis tends to be slightly lower than that of the fibres with the demarcation line along the major axis, and that, as in other normal wools of like fineness, the percentage of paracortex is the lesser.

TABLE 4

MEAN THICKNESSES OF GROUPS OF ANOMALOUS MERINO WOOL SAMPLES FOR EACH TYPE OF SEGMENTATION AS GIVEN IN TABLE 3

Sample No.	Mean Thickness (μ)	Mean Thickness of Group (μ) for Each Type of Segmentation							
		<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	<i>F</i>	<i>G</i>	<i>H</i>
D2	21.8	—	—	—	—	—	—	—	21.8
D19	23.0	23.1	—	—	—	—	—	22.7	—
D30	23.4	22.8	22.7	24.2	—	—	25.4	26.7	—
D28	24.1	24.2	22.3	24.3	—	—	27.2	26.2	—
D10	24.4	24.6	—	23.2	30.5	—	26.5	24.5	24.5
D17	25.8	24.6	—	25.0	—	—	27.1	27.2	30.6
D25	26.4	24.4	20.4	26.1	26.0	35.0	30.1	27.0	—
D20	26.5	25.6	22.6	26.6	—	—	24.4	27.0	27.5
D11	26.6	25.3	—	27.3	26.0	—	—	30.0	25.1
D1	26.7	—	—	—	—	—	—	—	26.7
D29	26.8	25.2	26.4	27.8	—	—	27.6	23.7	—
D15	27.0	26.2	—	26.6	—	23.7	24.9	30.5	27.8
D4	27.8	23.7	24.9	25.5	—	—	29.4	29.4	27.9
D6	28.8	29.1	—	29.0	23.7	—	—	29.1	27.1
D7	28.3	21.8	—	26.2	27.6	34.5	29.5	31.0	30.1
D9	28.6	28.0	—	28.0	—	—	—	28.4	28.8
D23	29.0	28.0	19.2	29.4	—	—	29.5	29.2	31.2
D31	29.0	25.6	26.2	30.4	45.0	38.0	36.0	29.5	40.6
D8	29.6	27.0	—	27.3	—	—	34.0	30.0	35.1
D14	30.6	25.0	—	29.5	37.2	—	—	34.0	33.0
D12	33.3	30.2	—	32.9	—	—	37.5	35.2	44.0
D5	34.2	—	—	34.6	35.5	—	—	32.0	36.0
D18	34.3	—	—	31.6	—	—	—	30.5	34.5
D3	34.7	31.4	—	33.0	—	35.5	36.0	34.7	28.3

In Table 2, the types of segmentation in 14 samples of normal wool have been noted. These demonstrate that there is a marked tendency towards the *A* and *B* types throughout all except N6, which is a coarse Corriedale wool whereas the others in the group are of Merino type. If these details are compared with Tables 3, 4, and 5, the differences between normal and anomalous wools become apparent.

IV. DISCUSSION

While, on the average, the anomalous wools tended to be coarser than the flocks from whence they came, the real contrast lay in the types of bilateral cortical segmentation. In general, for normal wool up to $27\ \mu$ mean thickness, the segmentation revealed in the cross sections is well defined and almost wholly in the *A* and *B*

TABLE 5
NUMBERS OF ANOMALOUS MERINO WOOL SAMPLES FALLING INTO THE THREE THICKNESS RANGES
 $25\ \mu$ AND UNDER, $26\text{--}35\ \mu$, AND $36\ \mu$ AND OVER

Sample No.	Thickness Ranges																							
	25 μ and Under								26-35 μ								36 μ and Over							
	A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H
D2	—	—	—	—	—	—	—	40	—	—	—	—	—	—	—	10	—	—	—	—	—	—	—	—
D19	3	—	—	—	—	—	12	25	1	—	—	1	—	—	—	8	—	—	—	—	—	—	—	—
D30	16	14	5	—	—	1	1	—	2	2	5	—	—	1	2	—	—	—	—	—	—	—	—	—
D28	15	7	4	—	1	1	3	—	8	2	2	—	—	6	—	—	—	—	—	—	—	—	—	1
D10	10	—	10	—	—	—	9	6	4	—	2	2	—	1	2	4	—	—	—	—	—	—	—	—
D17	15	—	7	—	—	—	4	—	6	—	7	—	—	2	6	2	—	1	—	—	—	—	—	—
D25	6	2	11	2	—	—	3	—	2	—	9	2	1	5	7	—	—	—	—	—	—	—	—	—
D20	6	1	6	—	—	2	2	3	8	—	12	—	—	—	4	6	—	—	—	—	—	—	—	—
D11	7	—	—	1	—	—	—	16	5	—	5	1	—	—	2	13	—	—	—	—	—	—	—	—
D1	—	—	—	—	—	—	—	24	—	—	—	—	—	—	—	16	—	—	—	—	—	—	—	10
D29	5	1	6	—	—	3	1	—	10	2	16	—	—	4	2	—	—	2	—	—	—	—	—	—
D15	8	—	3	—	1	2	—	2	12	—	11	—	—	—	3	5	—	—	—	—	—	—	2	1
D4	1	1	3	—	—	—	1	6	—	—	2	—	—	1	13	21	—	—	—	—	—	—	—	1
D6	3	—	4	1	—	—	4	2	5	—	16	—	—	—	7	5	1	—	—	—	—	2	—	—
D7	5	—	7	2	—	—	4	—	1	—	6	3	2	4	7	3	—	—	1	—	—	5	—	—
D9	—	—	1	—	—	—	2	2	4	—	7	—	—	—	10	23	—	—	—	—	—	—	—	1
D23	2	1	4	—	—	—	—	—	5	—	12	—	—	3	15	5	—	—	1	—	—	1	1	—
D31	13	2	1	—	—	—	—	—	12	3	3	—	—	2	5	—	—	2	1	2	3	—	—	1
D8	1	—	4	—	—	—	4	—	5	—	10	—	—	1	15	6	—	—	—	—	—	—	1	3
D14	6	—	4	—	—	—	—	2	3	10	1	—	—	—	5	6	—	2	2	—	—	2	5	—
D12	—	1	—	—	—	—	—	1	16	—	4	—	—	2	3	—	—	3	—	—	5	5	1	—
D5	—	—	—	—	—	—	—	—	—	—	10	6	—	—	13	3	—	7	7	—	—	2	2	—
D18	—	—	—	—	—	—	—	—	—	—	1	—	—	—	2	31	—	—	—	—	—	13	—	—
D3	4	—	11	—	—	13	4	1	—	—	1	1	2	11	2	—	—	—	—	—	—	—	—	—
Totals	126	30	91	6	2	22	54	130	109	19	142	16	5	43	125	167	1	—	18	11	2	9	33	26

categories (Tables 1 and 2). It is obvious from Table 5 that the classifications *C-H*, which are more typical of coarser wools, are freely represented among fibres which are " $25\ \mu$ and under" in thickness, as well as in the " $26\text{--}35\ \mu$ " region. It would appear as though a factor, normally operating in the production of coarse fibres only, is also present in the growth of many of these anomalous or doggy wool fibres, even at a low fibre-thickness level. Some of the peculiar variants from the simple bilateral

segmentation of type *A* or *B* may well lead to explanations of peculiarities of fibre crimp form and physical response seen in these wools. Another deviation from normal growth appears to be a greater area of paracortex in the doggy samples as compared with that in normal wool growth (Tables 1, 2, and 3). In these cases 24 doggy samples averaged 55 per cent. paracortex while 14 normal samples had a mean as low as 37 per cent. These values are only indicative, as the assumption has been made that in all cases the methylene blue staining has been similar in degree and that the orthocortex has been clearly and completely defined.

V. CONCLUSIONS

Conclusions from this study are:

- (i) That doggy Merino wool, in general, appears to be coarser than the normally crimped production of the flock in which it originates.
- (ii) That the cortical segmentation of fine fibres from doggy wool may show characteristics normally seen only in much coarser-wooled breeds.
- (iii) That the area occurrence of paracortex appears to be higher in doggy wool than in normal Merino wool.

VI. ACKNOWLEDGMENT

The best thanks of one of the authors (N. A.) is due to the Colombo Plan Authorities for the opportunity of carrying out this research in Australia.

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THE RESPONSE OF THE YOUNG TOMATO PLANT TO A BRIEF PERIOD OF WATER SHORTAGE

III. DRIFTS IN NITROGEN AND PHOSPHORUS*

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[*Manuscript received November 1, 1956*]

Summary

The drifts in nitrogen and phosphorus for the whole plant, its principal parts, and the laminae and petioles of the individual leaves are considered for tomato plants subjected to a brief period of wilting after they had developed eight easily manipulable leaves. The aim was to estimate some of the internal factors which contributed to the growth response previously described.

Treatment effects on nitrogen and phosphorus content were the resultant of two differing trends, that during and that subsequent to wilting, but were expressed most strongly in the nutrient uptake of the more actively growing organs.

During wilting, the uptake of nitrogen and phosphorus was markedly reduced and both nutrients were translocated from the laminae to the stem. Upon re-watering, active uptake of both nutrients was resumed and both passed preferentially to the laminae rather than to the stem. Phosphorus was the more labile of the two nutrients and wilting depressed phosphorus content more than nitrogen content. It was concluded that this depression was initiated relatively early in the drying cycle and preceded the effect on dry weight. However, the uptake of phosphorus upon re-watering was less rapid than that of nitrogen.

All laminae shared in the drastic effects of water shortage but, whilst the upper laminae suffered the greatest absolute depression, they nevertheless retained the greatest capacity to resist the depressing effects of water shortage. The response of the younger parts points to their playing a dominant role in maintaining the organization of the plant when exposed to water stress.

Internal factors were primary in all these responses and factors of external supply were secondary. A mechanism is suggested for the development of nitrogen-rich plants under a succession of moisture shortages.

I. INTRODUCTION

Previous papers (Gates 1955*a*, 1955*b*) have shown that in their response to water shortage it was the younger portions of the total leaves of young tomato plants that were important centres for the development of the whole plant response. This response was described as a senescent decline in growth during wilting and the development of a physiologically younger condition upon recovery. The pattern of growth in this response is of interest in itself but it would be valuable to determine some of the internal factors which contributed to the observed response. In this paper, therefore, the drifts in nitrogen and phosphorus in response to treatment are considered for the whole plant and for the individual leaf parts.

* The work described in this paper formed part of a thesis for the degree of M.Sc. Agric. in the University of Western Australia.

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II. EXPERIMENTAL

The cultural details and procedure in harvesting for experiments 1 and 2 have been described in Part I of this series (Gates 1955a).

Total phosphorus was estimated by the colorimetric method of Zinzadze, as modified by Gerritz (1940), following wet digestion with sulphuric, nitric, and perchloric acids. Total nitrogen was estimated by the Kjeldahl procedure, using selenium as catalyst.

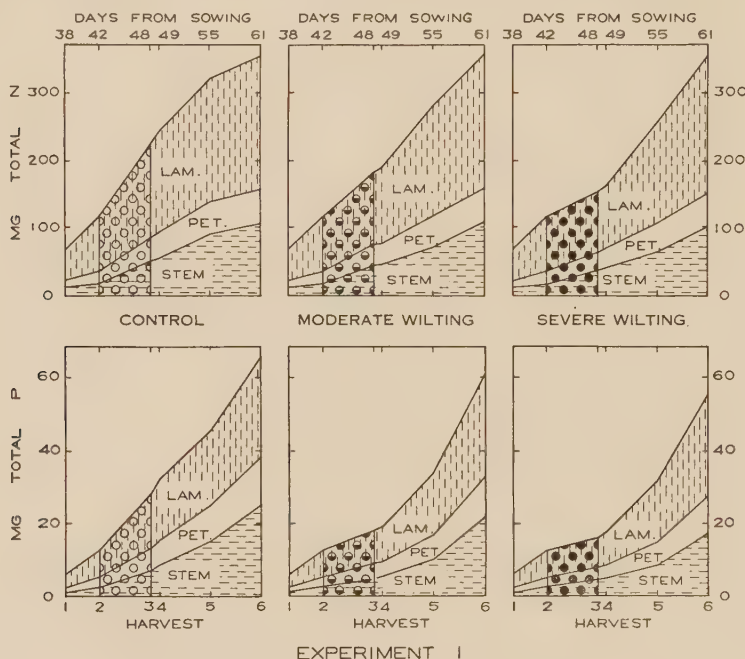


Fig. 1.—Absolute nitrogen and phosphorus contents of the laminae, petioles, and stem in response to treatment in plants of experiment 1. The data are plotted additively. The period of wilting is denoted by the blocks of circles and its severity by the intensity of their shading.

III. PRESENTATION OF DATA

As in the previous papers (Gates 1955a, 1955b), treatments will be referred to as:

C (control): frequent watering to field capacity

WM : brief period of moderate wilting

WS : brief period of severe wilting

The date of the two experiments will be presented separately in order to avoid confusion.

Experiment 1

(a) The Whole Plant and its Principal Parts

(i) *The Primary Data.*—The data relating to intake and distribution of nitrogen and phosphorus within the plant tops as affected by treatment are summarized in

Figure 1 where the absolute values for laminae, petioles, and stem are plotted additively. The numerical data are presented in Table 1.

TABLE 1
ABSOLUTE AMOUNTS OF TOTAL NITROGEN AND TOTAL PHOSPHORUS IN THE LAMINAE, PETIOLES,
AND STEM IN EXPERIMENT 1

Plant Part	Harvest	Nitrogen (mg/plant)			Phosphorus (mg/plant)		
		C	WM	WS	C	WM	WS
Laminae	1	45.5			3.87		
	2	79.4			7.44		
	3	137.0	106.9	88.1	14.75	8.98	8.03
	4	149.8	111.1	91.8	16.93	9.37	8.82
	5	179.4	162.9	149.9	20.90	16.96	16.70
	6	196.0	198.3	202.2	27.29	28.21	27.39
Petioles	1	11.0			1.15		
	2	19.5			2.62		
	3	35.3	30.9	28.0	6.15	3.90	3.42
	4	38.6	31.2	29.1	6.97	3.74	3.50
	5	50.0	47.7	42.1	9.53	6.53	6.04
	6	50.6	50.1	41.5	13.41	11.52	10.47
Stem	1	11.9			1.16		
	2	17.0			2.62		
	3	51.1	44.7	37.4	7.24	—	4.48
	4	55.2	45.8	41.2	8.35	5.68	5.10
	5	90.8	71.0	64.7	15.12	10.17	8.69
	6	107.2	108.3	100.2	24.86	21.33	17.14

In the control series the absolute nitrogen and phosphorus contents increase throughout the course of the experiment, in all plant parts. During wilting, the levels of absolute phosphorus and nitrogen are depressed in both WM and WS relative to C, the depression being relatively greater for phosphorus. Following wilting there is a reversal of these trends in both WM and WS, so that by the final harvest (13 days after re-watering), the values for absolute nitrogen in the plant tops are approximately equal in all treatments, whilst those for phosphorus have risen more steeply in the wilted plants than in C.

The data of Table 1 show that the major plant parts respond to treatment in a fashion generally similar to the plant tops. Absolute levels of nitrogen and phosphorus are depressed by the water shortage in all fractions, the effects being more severe in WS. As in the plant tops, phosphorus levels in the principal parts are depressed relatively more than nitrogen, and the effect on phosphorus in the laminae and petioles for WM is almost as great as for WS. After wilting, the tendency to regain control levels by the final harvest is most pronounced in the laminae and

least in the stem, for the laminae regain control level, whilst the stem does not. Stem phosphorus in both WM and WS is well below C at the final harvest, but stem nitrogen is only below in WS. The response of the petioles is intermediate to that of the stem and the laminae both during and after wilting. It is obvious that despite a general similarity to the trends for the plant tops there are differences between the fractions in their response. These differences will be discussed more fully below.

TABLE 2

RELATIVE AMOUNTS OF TOTAL NITROGEN AND TOTAL PHOSPHORUS IN THE LAMINAE, PETIOLES, AND STEM IN EXPERIMENT I

Plant Part	Harvest	Nitrogen (per cent. dry wt.)			Phosphorus (per cent. dry wt.)		
		C	WM	WS	C	WM	WS
Laminae	1	5.88			0.500		
	2	6.17			0.578		
	3	6.12	5.76	5.42	0.659	0.484	0.494
	4	6.06	5.62	5.58	0.685	0.474	0.537
	5	5.13	5.40	5.48	0.598	0.563	0.611
	6	4.39	4.78	5.35	0.611	0.681	0.724
Petioles	1	4.44			0.462		
	2	4.16			0.561		
	3	3.75	3.61	3.76	0.654	0.455	0.459
	4	3.66	3.48	3.92	0.661	0.417	0.473
	5	2.96	3.44	3.48	0.564	0.472	0.499
	6	2.12	2.40	2.94	0.562	0.551	0.599
Stem	1	4.65			0.453		
	2	3.14			0.483		
	3	4.16	3.59	3.76	0.589	—	0.450
	4	3.98	3.51	3.87	0.602	0.435	0.479
	5	3.18	3.16	3.63	0.529	0.452	0.488
	6	2.07	2.33	2.82	0.480	0.459	0.482

The data for the relative amounts of nitrogen and phosphorus are presented in Table 2. The relative nitrogen contents of the plant parts decline during the course of the experiment. Relative phosphorus contents, on the other hand, rise steeply until harvest 3 and 4 and then fall with time.

Both the laminae and the stem show marked depressions in relative phosphorus due to wilting, but upon re-watering the values are restored to above C, especially in the laminae. In the petioles, nitrogen levels are soon restored upon re-watering but phosphorus levels are not restored until the final harvest in both WM and WS.

(ii) *Rates of Accumulation.*—The absolute and relative data for the plant tops indicate that wilting, whether in a moderate or a severe form, caused a rapid decline in the accumulation of both phosphorus and nitrogen, but especially of

phosphorus. It has already been shown (Gates 1955a) that growth was reduced during wilting relatively early in the drying cycle. It can be concluded therefore that the influx of these nutrients was either impaired in the early stages of water shortage, that nutrient taken up in the early stages was lost again at later stages of the water shortage, or that both occurred. By the time the soil had dried to the vicinity of the permanent wilting percentage, the effects on accumulation of these nutrients had become quite drastic.

Although the contents of nitrogen and phosphorus were reduced in the plant tops, it is conceivable that these nutrients were absorbed and were stored in the roots as the wilt progressed. However, it has been shown (Gates 1955a) that wilting had drastic effects on the development of the root system, and it would perhaps be unlikely that root systems which were impaired in their development would become sinks for enhanced quantities of nutrients.

Further light can be thrown on this question by using the procedure suggested by Williams (1948) for calculating rates of intake per unit weight of root.

Mean values for I_M , the instantaneous rate of intake of a nutrient, were calculated for the wilting period using the approximate formula

$$I_M = \frac{\log_e R_2 - \log_e R_1}{t_2 - t_1} \times \frac{M_2 - M_1}{R_2 - R_1},$$

where R is the dry weight of the root system at each harvest occasion and M the nutrient content in the plant tops* (see Table 3).

TABLE 3
RATE OF INTAKE OF NITROGEN AND PHOSPHORUS PER UNIT ROOT WEIGHT

Nutrient	C	WM	WS
Nitrogen (mg/g/day)	33.9	23.4	14.1
Phosphorus (mg/g/day)	4.9	1.9	1.2

The above considerations suggest that neither the amount of nitrogen and phosphorus absorbed nor the rate of their absorption were increased by the wilt treatments which were imposed.

The comparisons for the single-day harvest interval 3-4 cannot be regarded as being established with as great precision as the data for other intervals, but they nevertheless give useful indications of the immediate effects of re-watering. By comparison with C, there is little change in absolute levels of nutrient content in WM and WS for at least 24 hr. The relative contents show slight increases for WS but slight decreases for WM in both nutrients. Very little is contributed to the overall trends in nutrient content of the post-wilt period.

* Since the root systems make up less than 15 per cent. of the dry weight of the plants, the omission of root nitrogen and phosphorus could scarcely alter the picture.

The rise in absolute nitrogen for the subsequent harvest periods is large by comparison with C, especially for WS, but phosphorus increments are not greatly larger than C. The increments after wilting are as given in Table 4.

TABLE 4
INCREMENTS IN NITROGEN AND PHOSPHORUS CONTENTS AFTER WILTING

Treatment	Nitrogen (mg)		Phosphorus (mg)	
	Harvest Interval		Harvest Interval	
	3-5	5-6	3-5	5-6
C	96.9	33.4	17.4	20.0
WM	98.9	75.1	15.7	27.4
WS	103.0	97.2	15.5	23.6

The increment for nitrogen is large by comparison with C mainly because of the small control increment at the final harvest interval. This suggests that the

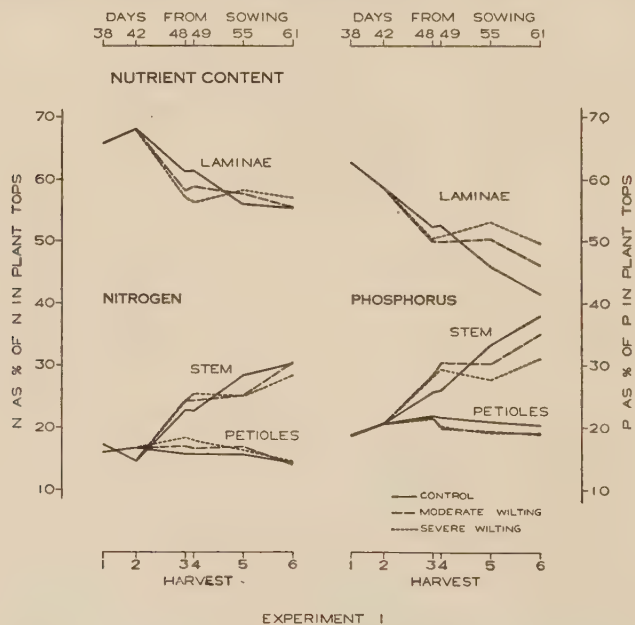


Fig. 2.—Proportions of nitrogen and phosphorus in the various fractions of the plant tops, expressed as a percentage of the total. Data for experiment 1.

supply of nitrogen may be limiting at this stage, so shedding some doubt on the control values at the final harvest, but it should be noted that the relative nitrogen contents are still normal for plants of that size at the conclusion of the experiment.

(iii) *Distribution between Major Plant Parts.*—The data for the proportions of the total nitrogen and phosphorus in the various fractions of the plant tops, which are presented in Figure 2, show that there are marked differences in the responses of the stem and the laminae. During wilting, the proportion of each element in the laminae falls by comparison with C and that for the stem rises. But, during recovery, this trend is reversed, for the laminae contain the greater proportion soon after wilting whereas the stem contains the lesser. The petioles seem to show very little response. These trends are of greater magnitude for phosphorus than they are for nitrogen. It has already been observed (Gates 1955*a*) that, during wilting, dry matter is translocated from the laminae to the stem, although it could not be established that translocation back again occurred upon recovery. The data of Figure 2 suggest that in addition to dry matter, translocation of nitrogen and phosphorus from the laminae to the stem occurred as a response to wilting.

TABLE 5
INDICES OF DISTRIBUTION OF NUTRIENT BETWEEN LAMINAE, PETIOLES, AND STEM IN
EXPERIMENT I

Harvest Interval	Treatment	Nitrogen			Phosphorus		
		Laminae	Petioles	Stem	Laminae	Petioles	Stem
1-2 (prior to wilting)	C	71.4	17.8	10.8	54.8	22.6	22.5
2-3 (during wilting)	C	53.6	14.7	31.7	47.3	22.8	29.9
	WM	41.2	17.2	41.5	29.2*	24.2*	46.6*
	WS	23.1	22.7	54.1	18.3	24.4	57.2
3-5 (after wilting)	C	43.7	15.2	41.0	35.3	19.4	45.3
	WM	56.6	16.9	26.6	50.8*	16.7*	32.4*
	WS	60.0	13.7	26.5	55.9	16.9	27.2
5-6 (after wilting)	C	49.8	1.7	49.3	31.9	19.4	48.7
	WM	47.1	3.3	49.6	41.1	18.2	40.7
	WS	53.8	9.6	36.5	45.3	18.8	35.8

* Based on interpolation of the value for stem phosphorus at WM 3.

The proportions of these nutrients passing to the principal fractions in response to treatment may be seen more clearly if the concept of distribution indices, as used by Williams and Shapter (1955) for dry weight, be applied. Williams and Shapter expressed the increments in dry weight, for the several fractions of a plant for each interval, as percentages of the total dry weight increment for that interval. The increments of nitrogen and phosphorus are considered in the same way in Table 5, which presents indices of nutrient distribution within the tops, both during wilting and in the recovery period.

During the period of wilting, the indices are high for the laminae in C and low for the stem, but in WS, and even in WM, the reverse is the case. Upon re-watering, the indices show that both nutrients passed preferentially to the laminae rather than to the stem in both WS and WM. Indeed, the values for the laminae were consistently higher in WS than in C.

TABLE 6

INCREMENTS IN NITROGEN AND PHOSPHORUS IN THE LAMINAE AND STEM FOR SUCCESSIVE HARVEST INTERVALS IN EXPERIMENT 1

Increment per unit increment of dry weight (mg/g)

Nutrient	Plant Part	Treatment	Harvest Interval				
			1-2	2-3	3-4	4-5	5-6
Nitrogen	Laminae	C	64.9	60.4	52.0	29.0	17.1
		WM		47.5	36.4	49.6	31.2
		WS		26.5	101.4	53.3	48.5
Nitrogen	Stem	C	17.4	50.2	25.5	24.4	7.2
		WM		40.0	16.7	26.8	15.6
		WS		47.0	42.3	33.0	19.3
Phosphorus	Laminae	C	6.8	7.7	8.9	3.9	6.6
		WM		2.7	3.4	7.3	9.9
		WS		1.8	21.5	7.2	9.9
Phosphorus	Stem	C	5.0	6.8	6.8	4.6	4.2
		WM		3.5*	9.9*	4.8	4.7
		WS		4.3	6.9	5.1	4.6

* Values obtained by interpolation for stem phosphorus content at WM 3.

This pattern of nutrient distribution both during and after wilting is similar to that already shown for dry weight (Gates 1955a), but affords no indication of the relative intensities in the response. Table 6 presents the increments of nutrient per unit increment of dry weight for successive harvest intervals. It is apparent from the relative magnitudes of the effects of wilting on the values for the laminae and the stem, that the translocation of nitrogen and phosphorus occurred to an even greater extent than the translocation of dry matter both during wilting and in the intervals following wilting. Furthermore, the change following re-watering occurred as early as the single-day harvest interval 3-4, and was relatively large, although the actual increments involved have been seen to have been small.

(b) *The Individual Leaves*

(i) *The Relative Data.*—Figures 3 and 4 present the relative data for the nitrogen and phosphorus contents of the laminae and petioles of the first eight leaves and of the rest of the leaves as a group, and for the stem, on each harvest

occasion. The scales have been adjusted, for each figure, so that the general mean of the values for each plant part is the same.

In C, the contents of both relative nitrogen and relative phosphorus in the laminae of successive leaves increases in passing up the plant. In the petioles, only the phosphorus content consistently changes in this manner. The increases are more pronounced for phosphorus than for nitrogen.

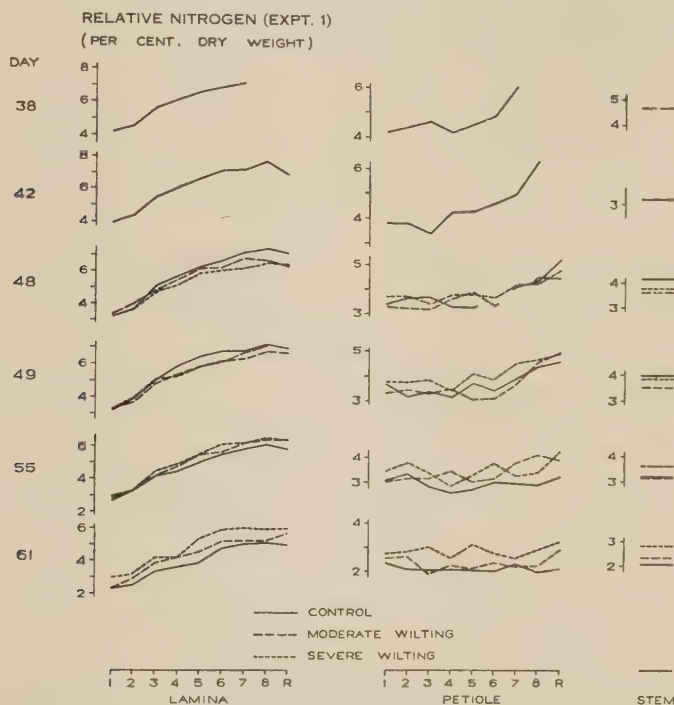


Fig. 3.—Relative nitrogen contents of the individual leaf fractions, the rest-of-the-leaves fractions (*R*), and the stem at various harvests. Data for experiment 1.

The nutrient contents of all leaf parts are affected and the effects are greatest in the upper leaves. The control pattern of increasing nutrient content in successive leaves is not greatly altered and the effects of WM are intermediate to those of WS and C in all the fractions.

The individual laminae reflect the treatment response already noted for the total laminae, but to differing degrees. The upper laminae are depressed most, relative to C, during wilting, and upon recovery, the upper laminae rise above C to a greater extent than the lower. The petioles of the upper leaves do not exhibit a greater treatment response than do those of the lower leaves, except in the case of relative phosphorus at harvests 3 and 4.

Both during and after wilting the individual fractions respond in a similar fashion but to a different extent, the actual degree of the response being related to the stage of development of the fraction.

(ii) *The Absolute Data.*—The absolute data for nitrogen and phosphorus contents of the individual leaf fractions are presented in Figures 5 and 6. The scales have been adjusted in the same manner as for the relative data.

In C there are similar trends with time in the nitrogen and phosphorus contents of the individual leaf fractions. The highest individual leaf values are found at approximately the sixth leaf, which may perhaps be associated with the fact that the first inflorescence appeared after the sixth leaf.

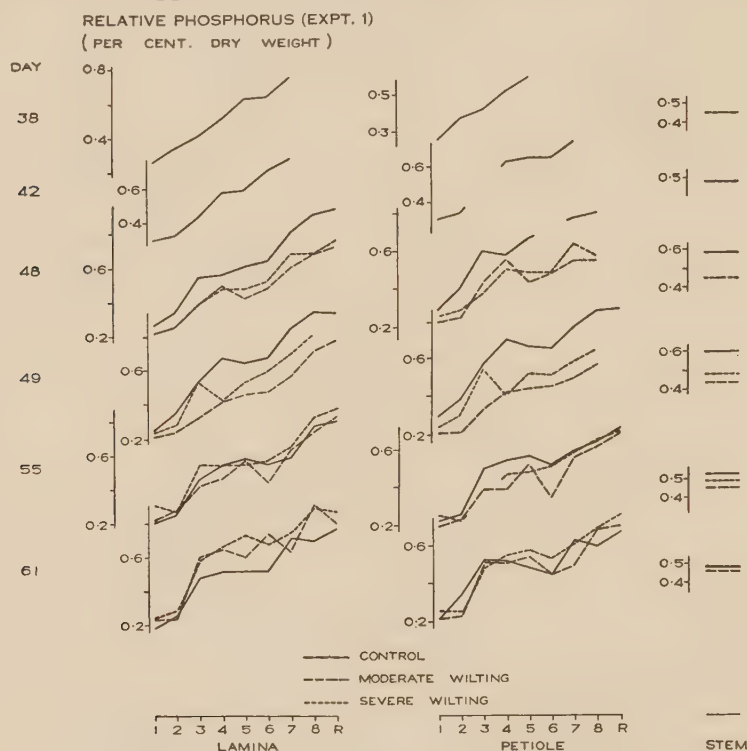


Fig. 4.—Relative phosphorus contents of the individual leaf fractions, the rest-of-the-leaves fractions (*R*), and the stem at various harvests. Data for experiment 1.

All laminae and petioles share in the pronounced treatment effects on nitrogen and phosphorus, but the effects are greatest in the upper leaves. This is to be expected from the effects of treatment on dry weight (Gates 1955*b*) and on the relative nutrient contents of the individual leaves.

During wilting, the restricted development of the leaf parts and the restricted content of these nutrients in both the absolute and the relative sense are greatest in the upper (younger) leaves. These effects are determined by the severity of wilting, except that phosphorus levels are more nearly similar in WM and WS than are nitrogen levels.

After wilting, the gradual regaining of control levels in the absolute values for the wilted plants occurs first in the lower leaf fractions, and later in the upper.

The degree of recovery in the upper laminae is such that control levels are virtually equalled, even exceeded in laminae 5 and 6. In the petioles, the recovery is not so pronounced for phosphorus. The petioles exhibit a response intermediate to that of the laminae and the stem.

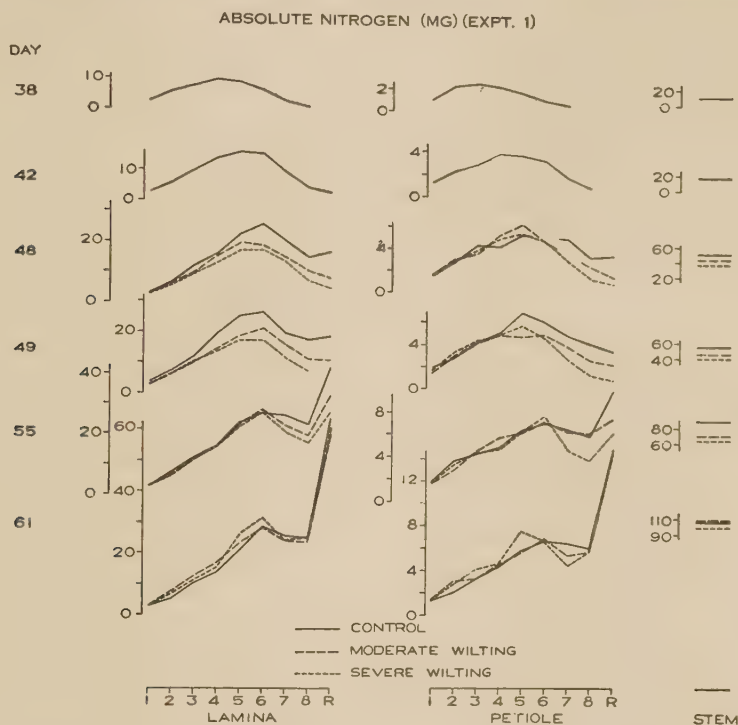


Fig. 5.—Absolute nitrogen contents of the individual leaf fractions, the rest-of-the-leaves fractions (*R*), and the stem at various harvests. Data for experiment 1.

A very evident feature of the data is that, despite the contrast in response to treatment during and after wilting, all fractions exhibit a similar pattern of response at any one time, although the intensity of the response differs. The treatment effects observed are always an expression of the response of the whole plant, and for any type of plant organ the degree of the response is related to the age of the fraction when treatment was imposed.

The pattern of response of the individual leaf fractions to moisture stress may be further described if the values for the harvest occasions at the time of wilting be examined within treatment. Figure 7 presents the logarithms of the nitrogen and phosphorus contents of the first eight laminae and petioles for harvest occasions 2, 3, and 4.

The control values increase greatly from harvest 2 to harvest 3 and, as is to be expected, only slightly from harvest 3 to harvest 4. During wilting, the depression in uptake of nitrogen and phosphorus is so marked in the laminae as to give very few individual increases. In fact, there is a slight loss of phosphorus and

possibly of nitrogen in the lower laminae, and only the uppermost laminae show real increases. The petioles show increases in all leaves for nitrogen, and in all but the lowest for phosphorus. These values suggest that the movement of nutrient from the laminae to the stem, which has been postulated above, was principally from the lower laminae. It should be noted that a similar conclusion has already been reached regarding the translocation of dry matter to the stem during wilting.

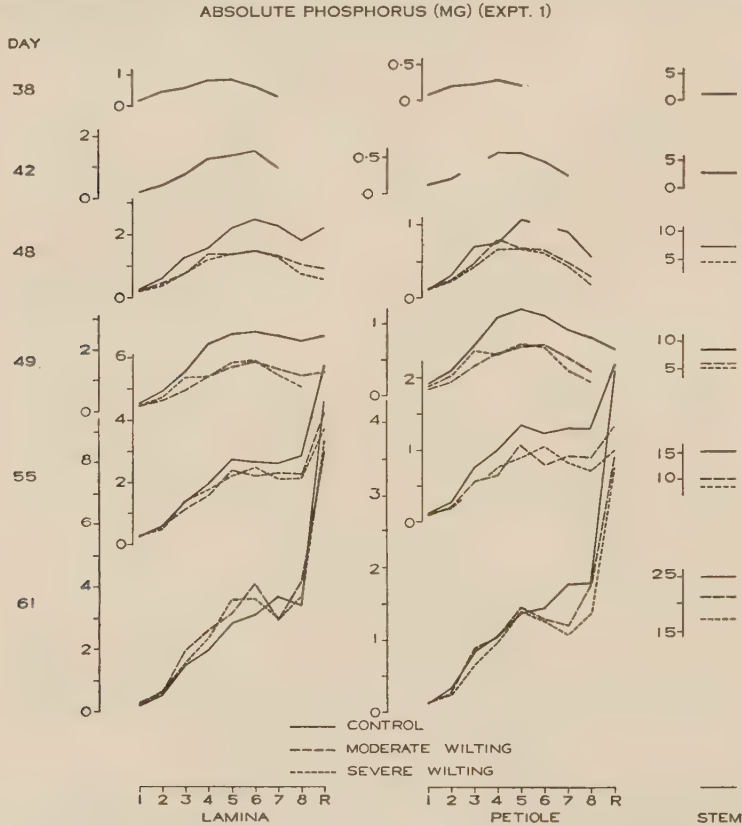


Fig. 6.—Absolute phosphorus contents of the individual leaf fractions, the rest-of-the-leaves fractions (*R*), and the stem at various harvests. Data for experiment 1.

It has been noted that the depression in nutrient uptake due to wilting was of greatest absolute magnitude in the upper laminae and least in the lower, being related to their rates of development. However, the small increments which did occur were principally in the uppermost laminae, and were slight or even negative in the lowest. All laminae, therefore, shared in the drastic effects of water shortage, but the upper laminae, whilst suffering the greatest absolute depression, still retained the greatest capacity to maintain their nutrient status.

The capacity to maintain active functioning might well be expected to be influenced by the degree of moisture stress imposed. The data of Figure 7 show that in both fractions of at least the oldest leaf, a loss of both nutrients occurred upon

re-watering of WM between harvest 3-4 but that this did not occur in WS. The interpretation of such small, but consistent differences requires caution, but they suggest that, upon re-watering, the uppermost laminae in WM were able to draw on nutrient reserves, even those of the older laminae, sooner than was the case with more severe wilting.

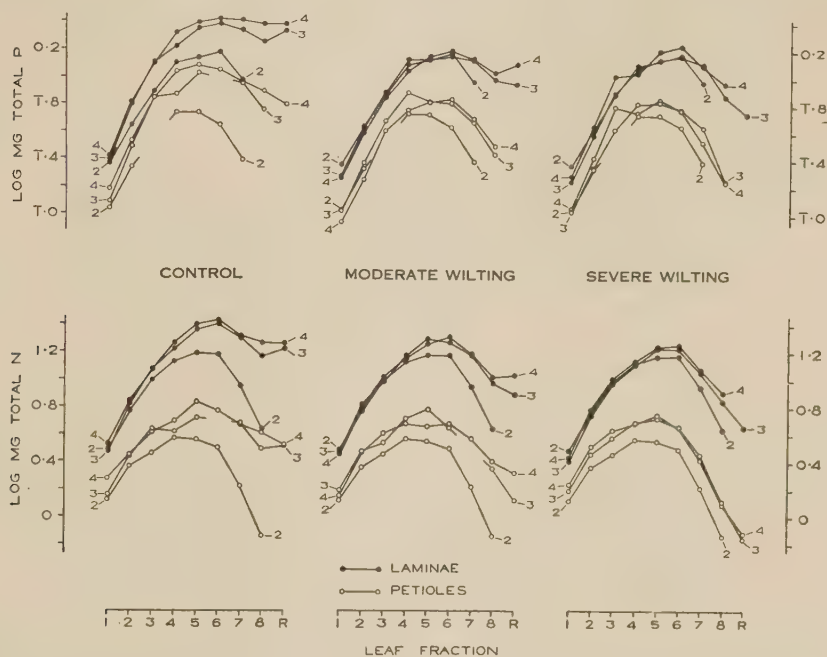


Fig. 7.—Log nitrogen and phosphorus contents (mg) of the laminae and petioles in experiment 1, grouped into treatments. Harvests are denoted by numerals, the comparison being for the second, third, and fourth harvests only.

These considerations make it clear that although the more actively developing younger leaf parts suffered the major check to their nutrient uptake this does not mean that their ability to maintain active functioning was completely lost. Rather, the capacity to resist the depressing effects of moisture stress on nutrient uptake by maintaining some degree of active synthesis seems to have been greatest in the younger leaf parts, and least in the older parts.

Experiment 2

The data of this experiment will not be presented as fully as that for experiment 1, owing to the similar character of the two experiments.

(a) The Whole Plant and its Principal Parts

Figure 8 presents the absolute values of phosphorus and nitrogen for the laminae, petioles, and stem plotted additively in a similar manner to the data for experiment 1 in Figure 1. The values for the inflorescence, which is normally included in the stem fraction, are indicated at harvest 6.

A feature of the data is the much higher absolute contents of both phosphorus and nitrogen in the plants of this experiment when compared with those of experiment 1. This is to be expected because of the greater size of the plants, but it is apparent from the data that, despite these size differences, the treatment response is generally similar.

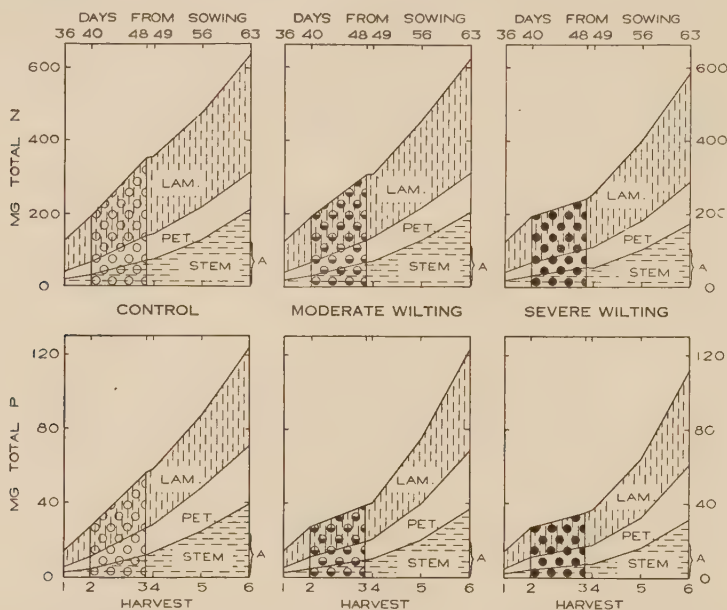


Fig. 8.—Absolute nitrogen and phosphorus contents of the laminae, petioles, and stem in response to treatment, in plants of experiment 2. The data are plotted additively. The period of wilting is denoted by the blocks of circles and its severity by the intensity of their shading. A, contents of the inflorescence at harvest 6.

During wilting, the absolute depressions of phosphorus and nitrogen in the plant tops in this experiment are greater than in experiment 1. This may be due partly to the greater plant size and partly to the longer time period involved in the development of similar degrees of moisture stress. The data are not presented, but these depressions are more similar in the two experiments if expressed either as increments per day or as increments per unit increment in dry weight. In experiment 2, the degree to which values are depressed is greater for phosphorus than for nitrogen in WM, it being almost as great as in WS.

Following wilting, the tendency in both WM and WS is to regain control level, as was the case in experiment 1, although at the final harvest, phosphorus more nearly approaches C, and nitrogen values are not equal for the three treatments. These trends were no doubt influenced by the additions of nutrient after re-watering in experiment 2.

The relative data for the plant tops will not be presented in full, the trends being generally similar to those of experiment 1. At the conclusion of wilting the depression for nitrogen was slight, but for phosphorus it was quite considerable.

The relative and the absolute values for phosphorus in the major plant parts at harvest 3 are given in Table 7.

TABLE 7
RELATIVE AND ABSOLUTE VALUES FOR PHOSPHORUS IN THE MAJOR PLANT PARTS
Data for harvest 3

Plant Part	C		WM		WS	
	Phosphorus (%)	Phosphorus Content (mg)	Phosphorus (%)	Phosphorus Content (mg)	Phosphorus (%)	Phosphorus Content (mg)
Laminae	0.750	29.41	0.586	19.15	0.647	17.59
Petioles	0.755	14.30	0.614	9.98	0.691	9.17
Stem	0.630	12.31	0.524	9.59	0.555	8.13

The absolute values for WM are intermediate to C and WS, but the relative values for WM are lower than for WS in all plant parts. Similar effects were observed for the laminae and petioles of experiment 1 and also for the majority of the individual leaf data. The effect is obviously a real one for both experiments and will be discussed later.

(i) *Rates of Accumulation*.—The depressions in nutrient intake by the plant tops during wilting suggest that in this experiment also, nutrient intake by the plant as a whole was reduced. The values for nutrient intake per unit weight of root during wilting have been calculated (see Table 8) in the same manner as for

TABLE 8
RATE OF INTAKE OF NITROGEN AND PHOSPHORUS PER UNIT ROOT WEIGHT

Nutrient	C	WM	WS
Nitrogen (mg/g/day)	24.8	18.5	8.8
Phosphorus (mg/g/day)	4.6	1.9	1.4

experiment 1. Wilting markedly depressed the values when based on the increments in the plant tops alone. Unfortunately no reliable estimates of nutrient content in the roots when wilted are available, but at harvest 4, 24 hr after re-watering, the absolute contents were as in Table 9.

For the roots to have behaved differently from the tops during wilting, viz. to have been sinks for nutrient, considerable amounts of nutrient would have had to pass to the tops during the harvest interval 3-4. This did not occur for phosphorus (increment in tops for C, 2.1 mg; WM, 0.91 mg; WS, 1.78 mg) but may have occurred for nitrogen in WS (control increment, 3.5 mg; WM, 1.7 mg; WS, 12.5 mg). However, in view of the shortness of the interval, there can be no certainty that the increment

of nitrogen in WS was due to translocation of nutrient absorbed by the roots during wilting. Such an increment did not occur in experiment 1. The data, therefore, suggest that both the roots and the tops were restricted in intake of nitrogen and phosphorus during wilting.

TABLE 9
ABSOLUTE NITROGEN AND PHOSPHORUS CONTENT OF ROOTS 24 HR AFTER
RE-WATERING

Nutrient	C	WM	WS
Nitrogen (mg)	40	38	35
Phosphorus (mg)	7.3	5.2	4.9

After re-watering, nutrient was added on two occasions (Gates 1955a) in order to ensure that the effects observed in experiment 1 were not the result of nutrient deficiency. The increments in the plant tops are as given in Table 10.

TABLE 10
INCREASES IN NITROGEN AND PHOSPHORUS CONTENT OF PLANT TOPS

Treatment	Nitrogen (mg)		Phosphorus (mg)	
	Harvest Interval		Harvest Interval	
	3-5	5-6	3-5	5-6
C	124.3	157.7	31.6	37.0
WM	149.5	171.4	36.4	48.5
WS	159.5	183.8	29.0	48.6

The increments in nitrogen are large for both WM and WS. Phosphorus values are highest for WM after wilting, but WM and WS are both high for the final interval. The picture is very similar to that of experiment 1, but the effects of additional nutrient are evident in the absence of a falling off in the rates of intake of nitrogen even in the control.

(ii) *Distribution between Major Plant Parts.*—Table 11 presents the data for the proportions of nitrogen and phosphorus in the several fractions of the plant tops, at the conclusion of wilting and at the conclusion of the experiment. The values are depressed in the laminae but rise in the stem during wilting, but by harvest 6 the reverse is the case. The effects are not as great as in experiment 1.

These trends are made especially clear by the data of Table 12 which presents the indices of nutrient distribution for the laminae and stem during the interval of wilting and the two intervals after re-watering.

Strongly contrasting trends between the laminae and stem are evident for the distribution of nutrient both during and after wilting. The data of Tables 11 and 12 suggest that translocation of both nutrients from the stem into the laminae occurred during wilting and was followed by their preferential distribution to the laminae upon recovery.

TABLE 11

PROPORTIONS OF NITROGEN AND PHOSPHORUS IN PLANT PARTS IN EXPERIMENT 2 (PER CENT. OF TOTAL IN TOPS)

Nutrient	Treatment	Harvest 3			Harvest 6		
		Laminae	Petioles	Stem	Laminae	Petioles	Stem
Nitrogen	C	60.3	19.0	20.7	50.5	16.1	33.4
	WM	57.5	20.2	22.3	50.1	17.1	32.8
	WS	56.4	20.9	22.7	50.7	19.6	29.8
Phosphorus	C	52.5	25.5	22.0	42.5	25.9	31.5
	WM	49.5	25.8	24.8	44.7	25.4	29.9
	WS	50.4	26.3	23.3	46.1	25.9	28.0

TABLE 12

INDICES OF DISTRIBUTION OF NUTRIENT BETWEEN LAMINAE AND STEM ONLY IN EXPERIMENT 2

Harvest Interval	Treatment	Nitrogen			Phosphorus		
		Laminae	Petioles	Stem	Laminae	Petioles	Stem
1-2 (prior to wilting)	C	60.5	23.3	16.2	53.4	27.9	18.7
2-3 (during wilting)	C	54.8	18.5	26.8	46.9	27.1	26.0
	WM	44.9	21.6	33.6	28.2	30.3	41.5
	WS	23.8	26.6	49.6	21.9	34.8	43.4
3-5 (after wilting)	C	36.8	18.0	45.2	31.8	29.6	38.6
	WM	42.7	17.0	40.3	45.5	25.1	29.3
	WS	52.1	18.2	29.6	48.1	24.6	27.3
5-6 (after wilting)	C	39.4	8.2	52.4	36.5	23.5	40.0
	WM	43.3	11.7	45.0	40.4	25.2	34.4
	WS	41.8	19.0	39.3	41.9	26.3	31.7

(b) *The Individual Leaves*

The absolute and relative data for the phosphorus and nitrogen contents of the laminae and petioles of leaves 2 and 6 are presented in Figure 9.

The fractions of leaf 6 are depressed during water shortage much more than those of leaf 2, the effect being arithmetically greater in leaf 6. Especially is this so for the absolute data. The effects for phosphorus tend to be greater than for nitrogen.

The more moderate effects of the water shortage on nitrogen contents must be considered in relation to the loss of dry matter noted previously (Gates 1955a, 1955b). It would appear that in this experiment the degree of translocation of nitrogen from the laminae to the stem during wilting was very similar to that of dry weight. The small and sometimes positive effects of wilting on the relative

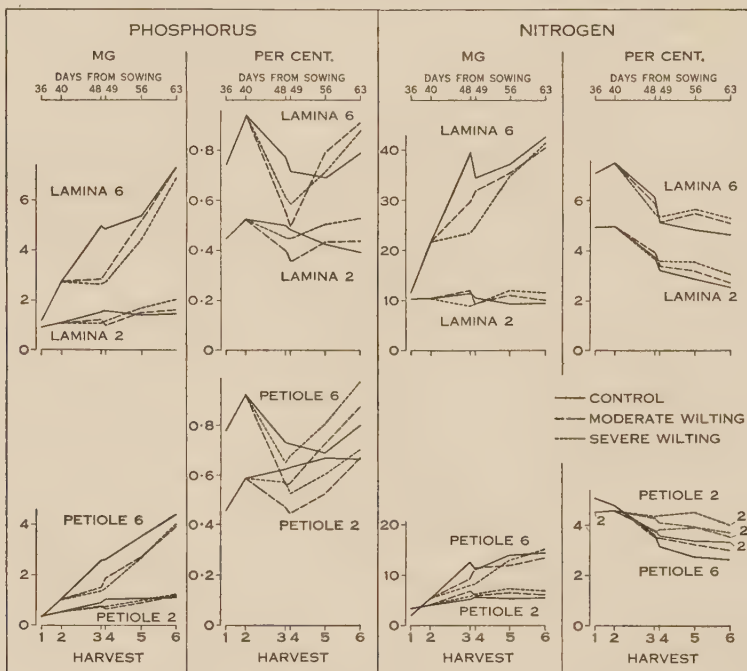


Fig. 9.—Primary data for the nutrient contents of the laminae and petioles of leaves 2 and 6 in experiment 2.

nitrogen contents indicate that the translocation of dry matter must sometimes have been relatively faster than that of nitrogen. In organs losing nutrient by translocation and losing dry matter by translocation and by respiration, the particular stage in treatment at which harvest occurs will greatly affect the relative nutrient contents.

As was pointed out earlier (Gates 1955b), the delay of 4 hr in harvesting the plants of this experiment at harvest 4 gave rise to misleading dry weight values. These affect the reliability of the relative data at this harvest for both phosphorus and nitrogen.

By the final harvest, absolute levels of the nutrients were generally above C in leaf 2 fractions and had almost attained control levels in leaf 6. Relative levels were markedly above C especially in leaf 6 fractions.

The data for the upper and lower leaf groups will not be presented as the individual leaf fractions are typical of them. Water shortage affected the nutrient contents in the younger parts more markedly than in the older parts during moisture stress, but upon re-watering recovery was more rapid in the younger.

IV. GENERAL DISCUSSION

(a) *Drifts in Nitrogen and Phosphorus during Moisture Stress*

(i) *Internal Factors.*—The data for both experiments show that during wilting translocation of both phosphorus and nitrogen took place from the laminae to the stem. This was usually more intense than the loss of dry matter from the laminae and was most pronounced for phosphorus. These changes during water shortage must have developed progressively as the water shortage developed, and at first the control pattern would have been followed. Thus the laminae would first have received more nutrient than the stem, and later this trend would have been reversed. The fact that WM caused effects so great as to be at best intermediate to C and WS suggests that a reversal of the normal trend must have occurred relatively early in the drying cycle. Indeed, for phosphorus, this must have commenced at a soil moisture content comparatively high in the available range.

For these changes to have occurred during water shortage suggests that the normal tendency to synthesis of soluble nitrogen and phosphorus into more highly organized compounds may have been checked, especially in the older laminae, thus giving rise to an increasing proportion of hydrolytic breakdown products. Maximov (1941) has claimed that water shortage leads to an enzymatically produced intensification of hydrolytic decomposition dependent on the physical state of the cell enzymes, and Petrie and Wood (1938) have shown that the net rate of protein hydrolysis increases with reduction of leaf water content. Such hydrolytic changes in the individual laminar fractions would be a reversal of their normal metabolic state, reminiscent of a senescent change, as has already been suggested for the effects of treatment on dry weight of these organs (Gates 1955*b*).

Williams and Shapter (1955), in discussing the data of Miller and Duley, have drawn attention to the probability that those parts which are growing most actively during a period of low water treatment are those which receive the greatest check to their growth. In the present case, whilst this has been shown for both dry weight increment (Gates 1955*b*) and nutrient content, nevertheless, the capacity to maintain active synthesis was least affected in these parts, and was most disturbed in the older parts which were growing more slowly. Clearly, any check to growth is likely to be of greatest total amount for the most actively growing parts, but that may not involve the complete discontinuance of active synthetic processes. In the experiments described here, the response of the younger parts points to their playing a dominant role in maintaining the organization of the plant when exposed to water stress. This would seem to be, then, an extension of the normal tendency of young and especially meristematic tissues to play a dominant role in the control of growth processes. It was pointed out by Williams (1948), with respect to phosphorus metabolism, that the juvenile tissues are largely involved in the control of the movement of carbohydrate reserves and mineral nutrients within the plant. It appears that, in the tomato, such a role is maintained even under water stress.

These considerations emphasize the point that the response to water stress presents an integrated pattern characteristic of the whole plant as an organism. In so far as this pattern may be modified in different plants, their drought resistance and their capacity to stand up to brief periods of water shortage, may be in part explained. Williams and Shapter (1955) have suggested that the capacity of juvenile tissues to continue to operate on a restricted supply of carbohydrates may be a characteristic of drought resistance. To this may be added the value of a capacity to delay the transfer of nutrients from the laminae to the stem under water stress. Such a capacity would delay the "haying off" of pasture plants under drought conditions (especially temporary drought) and would constitute a character of potential value to the plant breeder.

(ii) *The Relation to External Supply of Nutrients*.—Various workers (Petrie and Arthur 1943; Morton and Watson 1948) have suggested that a relatively greater uptake of nitrogen occurred from dry soil during low moisture treatment. Wadleigh and Richards (1953, p. 437 and 441) in reviewing the literature have concluded that "Most experimental evidence shows that for a given level of fertility, decreasing soil moisture supply is associated with a definite increase in nitrogen content of the plant tissue . . ." They also suggest that the "usually observed accumulation of nitrogen in plants on dry soils" may be due, at least in part, to the relative concentration of nitrate ions in the outer layer of thin moisture films, as soil moisture decreases.

In the experiments reported here, the net uptake of nitrogen in the period of wilting in both experiments was greatly impaired. If anything, this tendency was less in the second experiment, where the relative nitrogen contents in some fractions were not greatly affected by wilting. Nitrogen intake was reduced, however, and it was suggested that the effect on relative content was then largely due to the particular stage during or after wilting at which harvest occurred.

There are few, if any, cases in the literature where it has been shown that nitrogen uptake was increased by the greater concentration of ions developing as soil moisture decreased. Apart from the difficulty of interpreting relative data for the wilted plant, the usual practice (as in the case of Morton and Watson) has been to draw conclusions from plants harvested after a succession of wiltings, and this involves returning the soil to field capacity. Petrie and Arthur's data following brief wilting periods is somewhat misleading, for their harvests were in each case taken several days after returning the plants to normal soil moisture levels.

Reference has been made to the lower values for relative phosphorus in WM than in WS even though the differences in absolute content were slight. This means that the effects of the moisture shortage on absolute phosphorus levels were therefore greater than on dry weight in WM, and must have commenced prior to the effects on dry weight increments, which it has been suggested (Gates 1955a) occurred relatively early in the drying cycle.

It may be inferred that the hydrolysis of phosphorus compounds may therefore have been a direct effect of water stress and have commenced early in the drying cycle.

The considerably reduced uptake of phosphorus by the roots may have resulted from an increased inorganic phosphorus content resulting from these effects in the manner suggested by Williams and Shapter (1955). The data give no direct indication of loss of nutrients from the roots during treatment, but, Katznelson, Rouatt, and Payne (1954) have shown that seedlings of tomato, soybean, barley, and oats, when wilted in sand or in sand-soil mixtures, liberated a variety of amino acids from their roots.

The effects on phosphorus contents which have been described show that the phosphorus nutrition of the plant during moisture shortage seems to have been even more independent of external supply than does its nitrogen nutrition.

(b) The Reponse upon Re-watering

It has been noted that upon re-watering there was a complete reversal of the trends observed during wilting. The preferential distribution of nutrient to the laminae was most intense in the younger laminae, and was accompanied (Gates 1955*a*, 1955*b*), by a higher relative growth rate and marked rises in water content. These changes in the plant as a whole, may be described as a return to a more juvenile condition.

The more ready uptake of nitrogen and phosphorus upon re-watering by all parts of the plant tops might be attributed to a complex of factors, both external and internal. Wadleigh and Richards (1953) have drawn attention to the variable effects of water treatment on the fixing power of different soils for phosphorus and also to the effects of such treatments on microbiological activity. In the present experiments there would certainly have been a rise of soil temperature during wilting, and this might well have influenced both the fixing power of the soil and microbiological activity. However, the very different nutrient additions after wilting in experiment 2, by comparison with experiment 1, might be expected to have profoundly influenced nutrient uptake as between the two experiments if external factors had played a dominant role. This has not been the case to any marked extent. The increments of nitrogen and phosphorus upon re-watering are similar in the two experiments in that, at each interval, nitrogen increments were higher than C in the wilted plant, but phosphorus increments tended to lag behind.

The factors controlling such absorption seem once again to have been those of internal demand, perhaps influenced by the tendency for re-utilization of the more labile nutrient, phosphorus, but they were not entirely unrelated to external supply.

The tendency for water shortage to produce nitrogen-rich plants may result from these more drastic effects of moisture stress on phosphorus nutrition. This type of plant usually develops after a succession of moisture shortages. Early in the development of stress, phosphorus metabolism is disturbed prior to that of nitrogen, but upon re-watering a larger proportion of nitrogen than phosphorus would be absorbed before the plant again suffered moisture stress. Accordingly, both during moisture stress and upon return to field capacity, the tendency would be to develop a nitrogen-rich plant.

(c) The Relation between Growth Attributes and Nutrient Content

Morton and Watson (1948) noted a very considerable increase in the net assimilation rate of sugar-beets receiving high nitrogen treatment when switched from low to high water supply. This they connected with the relatively high internal nitrogen content of the plants of the dry series. They suggested that the increased water supply stimulated carbohydrate synthesis, so resulting in increased protein synthesis and the renewal of leaf production at the apex of the plant.

An alternative suggestion might be that the more juvenile tissues of the plant had resisted the drastic effects of water shortage to the greatest extent and were therefore able to mobilize the supplies of available substrate for active synthesis of protein and an increased net assimilation rate. Indeed in the present experiments it has been shown that the younger tissues maintained a greater capacity for active synthesis. The suppression of active synthesis that occurred in all parts of the plant tops, and the translocation of dry matter and nutrient from organs engaged in active synthesis, the laminae, suggest that the supplies of available substrate were in excess of the capacity for their re-utilization during stress. Upon recovery, therefore, active growth might be expected in the more juvenile tissues, and was observed to occur.

However, the causal mechanisms involved in these effects are far from clear. As both net assimilation rate and nutrition were simultaneously affected, it would seem most profitable to examine the nature of this effect on the factor first impaired, the phosphorus nutrition. This seems the more necessary in view of the close association of phosphorus with all synthetic processes, including the formation of protein and the assimilation of dry matter. To assess the effects of moisture shortage on the phosphorus-containing intermediaries of metabolism would seem highly desirable.

V. ACKNOWLEDGMENTS

It is a pleasure to acknowledge the guidance of Mr. R. F. Williams, Division of Plant Industry, C.S.I.R.O., in all aspects of the work. Thanks are due to Mrs. B. Lee for technical assistance.

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RESPONSES OF *AVENA* COLEOPTILE SECTIONS TO HIGH CONCENTRATIONS OF AUXIN*

By N. G. MARINOS†

[Manuscript received December 5, 1956]

Summary

(i) The initial elongation rate of excised coleoptile sections is shown to increase with increasing concentration of indoleacetic acid (IAA). In supra-optimal concentrations (1×10^{-3} M) this initial phase is followed by shrinkage of the sections accompanied by loss of water and solid matter; the latter is quantitatively recoverable in the solution. The respiratory rate drops rapidly while the sections elongate in 1×10^{-3} M IAA.

(ii) It is suggested that the long-term inhibitory effect of high auxin concentrations on growth is due to secondary damage and shrinkage of the tissue and not to an immediate retardation of the growth processes.

(iii) Attention is drawn to the difficulty of applying the methods of enzyme kinetics to the analysis of auxin-induced growth and especially the narrow range of conditions under which the methods are valid.

I. INTRODUCTION

It is now well established that the rate of growth of excised sections of *Avena* coleoptiles is related to the concentration of auxin in the external medium. However, the rates of elongation are not always constant with time and the need for care in interpreting growth rates based on the means of values measured over long time intervals has been emphasized by Audus (1952). Bonner and co-workers (Foster, McRae, and Bonner 1952; McRae and Bonner 1952; McRae, Foster, and Bonner 1953) have proposed a method for the analysis of the action of auxins and anti-auxins which involves the use of enzyme kinetics and depends upon the accurate determination of the initial rate of elongation of *Avena* coleoptile sections. To determine initial rates these workers measured increases in length after 12 hr and claim that under their conditions the rates of elongation were constant with time at any given concentration of auxin; supra-optimal concentrations were found to cause a depression of the growth rate. In view of the importance of conclusions reached by this method (McRae and Bonner 1952) experiments were conducted in this Laboratory to define further the responses of coleoptile sections to varying concentrations of auxin with time. It must be admitted that the conditions specified by Bonner and colleagues were not reproduced in these experiments, but the results obtained emphasize the narrow range of conditions in which Bonner's methods are applicable and the need for rigidly standardized conditions if initial rates of growth are to be measured and compared.

* The work described in this paper formed part of a thesis for the degree of Ph.D. in the University of Adelaide.

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The work to be described here was completed at the time two other papers appeared dealing with some aspects of this problem. In the first paper Bennet-Clark and Kefford (1954) criticized Bonner's methods on the grounds that they were unable to obtain linear growth with time and questioned the validity of a measurement after 12 hr as an estimate of the true initial rate. In reply Bonner and Foster (1955) pointed out that Bennet-Clark and Kefford's criticism was unfounded because different experimental conditions were used from those found to be essential for the measurement of true initial elongation rates. Some of the points raised by these two papers will be discussed in the light of the work to be described here.

II. MATERIALS AND METHODS

Only general methods are described here; specific methods will be discussed in conjunction with individual experiments. This work was carried out with *Avena* coleoptile sections. Seed of *A. sativa*, var. Victory, was obtained in 1947 from Svalof, Sweden, and in 1949 a sample was sown at the Waite Institute farm. Seeds from that harvest, after selection for uniformity in size, were used in the experiments to be described.

After preliminary trials the following method of growing the plants was adopted. Seeds were dehusked, soaked in water for 2 hr, and then arranged on rectangular glass plates covered with filter paper. These plates were held at a 60° angle by brass holders resting in glass dishes, and water was added to a level 0.5 cm below the tip of the embryonic root. The dishes were covered with lids which were removed after 72 hr. The plants were grown in a dark-room kept at 24°C and exposed to red light for the first 24 hr of growth; the experiments were carried out in the same room unless indicated otherwise.

After 90–96 hr sections of uniform length (3 mm) were cut 3–4 mm below the tip of the coleoptile by means of a two-bladed cutter. Usually only one section was obtained from each plant, although in certain experiments where large numbers of sections had to be handled it was found necessary to cut two or three successive sections. In such instances the sections were randomized before use.

The sections were floated in 15 ml of test solution in a petri dish and allowed to grow in complete darkness at 24°C for periods specified in each experiment. As most of the experiments reported here deal with the time-growth relationship, the changes in length at various time intervals were determined on the same sample of sections. At each reading the sections were placed on a glass plate with sufficient solution to prevent desiccation, measured, and immediately returned to the petri dish. All manipulations and measurements were carried out under red light in the dark-room. The length of the sections was measured in terms of a 100-unit scale mounted in the eyepiece of a binocular microscope at a magnification of 5 diameters. The original mean length of these sections was 52.0 ± 0.5 units (about 3 mm); changes in length have been expressed as percentages of the original value.

With one exception only indoleacetic acid (IAA) was used in these experiments. This was obtained from British Drug Houses Ltd. All solutions were kept in a refrigerator at 4°C and renewed at least once a fortnight. These test solutions, for reasons that will be set forth in the discussion, were used unbuffered and without any additives such as sugars and salts.

III. EXPERIMENTAL RESULTS

(a) *The Time-Growth Relationship for Coleoptile Sections Exposed to Supra-optimal Concentrations of IAA*

Initially the time-growth relationship of coleoptile sections exposed to optimal and supra-optimal concentrations of IAA was followed on material obtained from plants of varying age and under different temperatures during the incubation of the sections. Although these conditions affected the detailed course of elongation, the shape of a curve for a given auxin concentration was characteristic. A typical experiment is shown in Figure 1 where sections were obtained from 94-hr-old plants and their elongation followed in water, $1 \times 10^{-5}M$, and $1 \times 10^{-3}M$ IAA at $24^\circ C$.

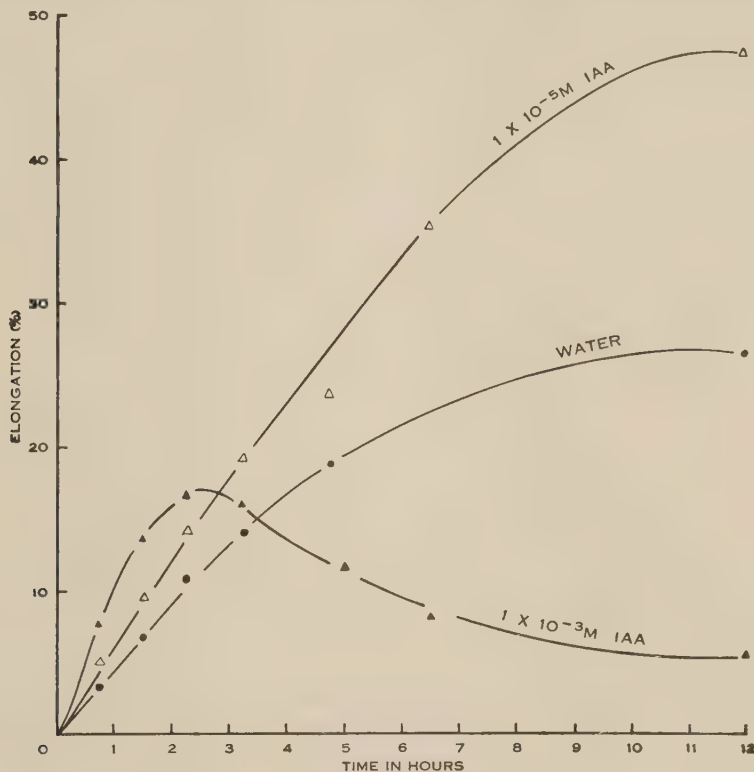


Fig. 1.—Growth-time curves for coleoptile sections growing in water, nearly optimal ($1 \times 10^{-5}M$), and supra-optimal ($1 \times 10^{-3}M$) concentrations of indoleacetic acid (IAA).

On the basis of the above data the characteristics of the response are as follows: (1) the initial rate of elongation in $1 \times 10^{-3}M$ IAA is greater than that in the optimal concentration of $1 \times 10^{-5}M$ IAA or water; (2) this initial rapid rate of elongation is constant for about $1-1\frac{1}{2}$ hr and then it falls off until at about $2\frac{1}{2}-3$ hr elongation ceases; (3) after the third hour the sections in high concentrations shrink and lose their normal coloration; (4) this shrinkage continues for about 3 hr after which time there is very little change in length; and (5) the extent of shrinkage in relation to the maximum elongation at the peak of the curve is fairly constant for any given set of conditions.

The behaviour of sections in a series of auxin concentrations is shown in Figure 2. In the three higher concentrations of auxin, $1 \times 10^{-2}M$, $3 \times 10^{-3}M$, and $1 \times 10^{-3}M$ IAA, the shape of the elongation curves is similar but with certain quantitative differences. With increasing concentration: (1) the rate of initial

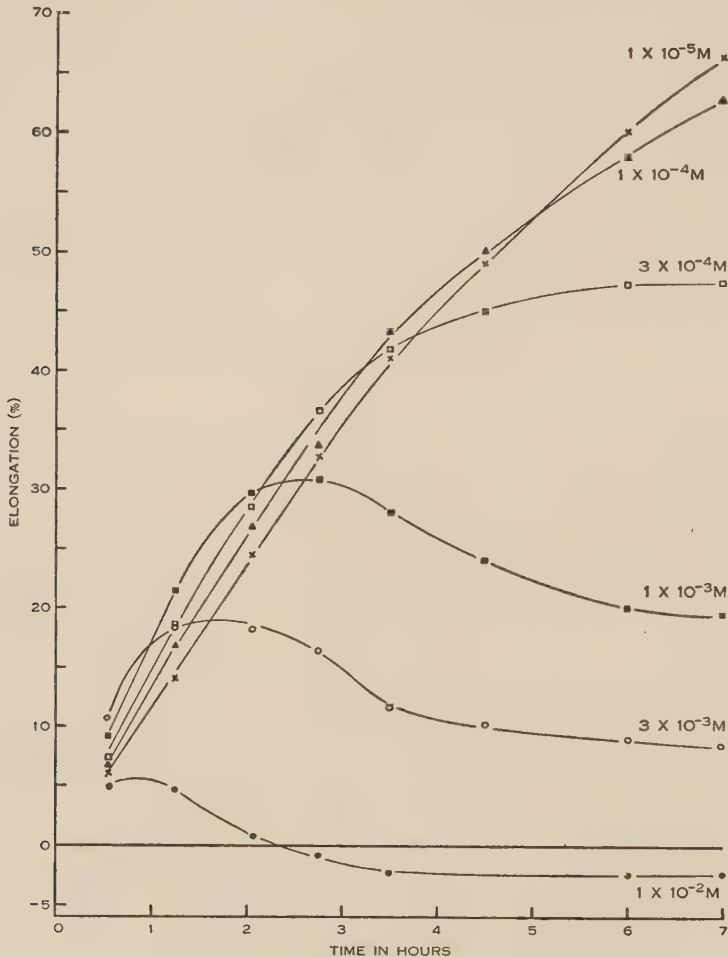


Fig. 2.—Growth-time curves for coleoptile sections over a wide range of indoleacetic acid concentrations.

elongation increases but lasts for a shorter time; (2) the maximum length of the sections before shrinking becomes smaller; (3) the peak of the elongation curve is reached sooner; and (4) the extent of shrinkage increases. Concentrations of $3 \times 10^{-4}M$ and below caused no shrinkage of the sections within the 7-hr period of this experiment.

Experiments of this nature established that the effect of high concentrations was a progressive reaction of the cells to an increasing concentration of auxin. The concentration of $1 \times 10^{-3}M$ IAA was chosen as the most practicable for further

studies because the sections elongated for a longer period of time and to a greater extent before shrinking than in the higher concentrations.

(b) *Conditions Affecting the Detailed Course of the Time-Growth Relationship*

Two conditions that alter quantitatively the response are age of the coleoptile and temperature during growth of the sections.

(i) *Effect of Coleoptile Age on the Response*.—Sections were obtained at the same time from plants of three different ages—52, 70, and 94 hr. Two concentrations of IAA were used: $1 \times 10^{-5}M$ and $1 \times 10^{-3}M$. In accordance with previous results of other authors the response of the sections to optimum concentration diminished with increasing age of the plants. In $1 \times 10^{-3}M$ with increasing age: (1) the maximum elongation, reached before shrinkage, decreased; these differences were small but reproducible; (2) the extent of subsequent shrinkage increased; (3) the maximum elongation was reached sooner; and (4) the rate of initial elongation was greater. It was therefore decided to use plants about 90 hr old for subsequent experiments.

(ii) *Effect of Temperature on the Response*.—This effect was examined by following simultaneously the elongation of sections in $1 \times 10^{-3}M$ IAA at three different temperatures—18, 23, and 28°C. The results indicated that: (1) the initial rate of elongation increased with increasing temperature—the Q_{10} value over the first hour was 1.94 between 18 and 23°C, and 1.91 between 23 and 28°C; (2) the time taken to reach maximum elongation was less the higher the temperature; and (3) maximum elongation was practically the same at all three temperatures. A temperature of 23–24°C was chosen as the most suitable for the purposes of this investigation.

(c) *The Time-Growth Relationship for Coleoptile Sections Exposed to Supra-optimal Concentrations of Auxins other than IAA*

This behaviour of coleoptile sections is not peculiar to high concentrations of IAA alone as shown by comparison with the responses to indolebutyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Fig. 3). The sections for these experiments were obtained from younger (75 hr old) plants since sections from 90-hr-old plants barely showed a response to $1 \times 10^{-3}M$ of both phenoxyacetic acids. All auxins were used in two concentrations: $1 \times 10^{-5}M$ and $1 \times 10^{-3}M$. There is practically no difference in the amount of elongation induced by the four substances when used at a concentration of $1 \times 10^{-5}M$. This confirms the findings of Muir, Hansch, and Gallup (1949). In $1 \times 10^{-3}M$ the response is quite different. In IAA the elongation curve conforms with what has already been described; in IBA the maximum elongation is about half as large and reaches the peak about half an hour earlier. The curves in 2,4-D and 2,4,5-T are identical and show only a small initial rapid extension for the first $\frac{1}{2}$ hr followed by a shrinkage to a point below the original length. The curve in these solutions is very similar to that obtained with $1 \times 10^{-2}M$ IAA (Fig. 2).

Therefore, the "high concentration effect" was not peculiar to IAA but the concentrations of other auxins giving similar responses differed. In all experiments that followed only IAA was used, although, in view of the results presented above,

it seems reasonable to assume that findings with IAA will also apply to 2,4-D, 2,4,5-T, and IBA.

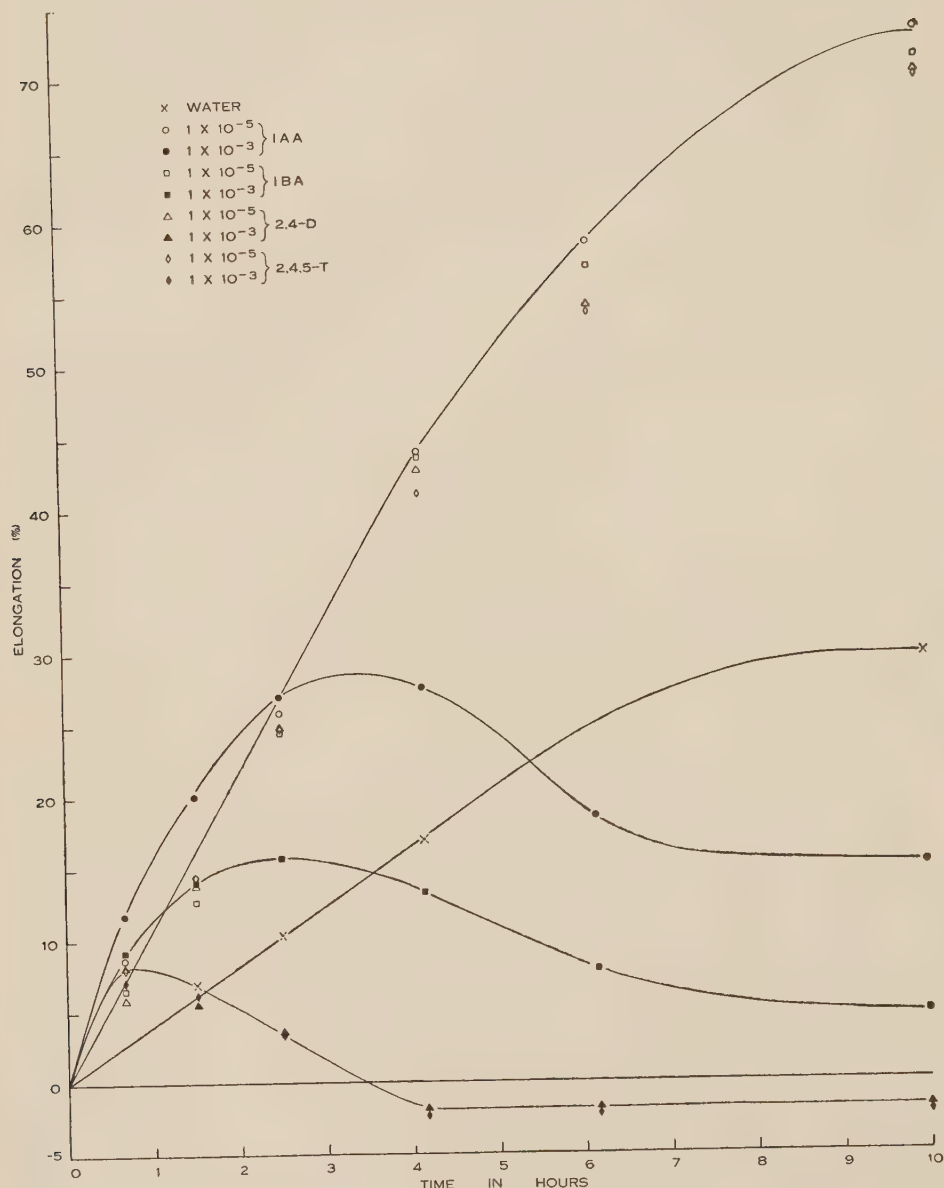


Fig. 3.—Growth-time curves for coleoptile sections in optimal and supra-optimal concentrations of four auxins—indoleacetic acid (IAA), indolebutyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T).

(d) *The Mechanism of Shrinkage of Sections in High Concentrations of Auxin*

The results presented above suggested that supra-optimal concentrations of auxin did not cause an inhibition of growth since their immediate effect was a

stimulation of elongation. However, this was followed by damage of the tissue. To find out whether secondary changes leading to shrinkage began during the initial elongation, sections were transferred to solutions of lower auxin concentration, or to water, and comparisons made with the elongation of sections kept continuously in high concentrations.

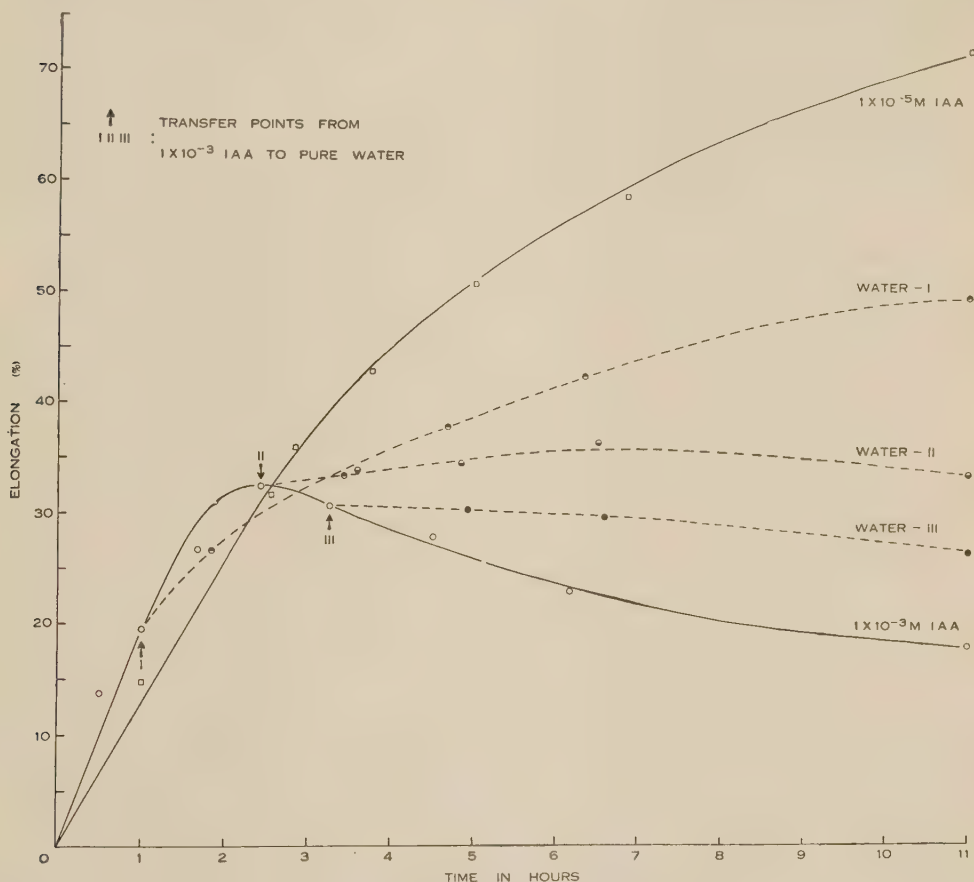


Fig. 4.—Growth-time curves for coleoptile sections transferred from 1×10^{-3} M indoleacetic acid (IAA) to water after 1, $2\frac{1}{2}$, and $3\frac{1}{4}$ hr.

Coleoptile sections were placed in a high concentration of auxin and random samples of 15 sections transferred at intervals to water. These samples were washed thoroughly during transfer. From the results (Fig 4) it is seen that if the transfers were made before the sections had reached their maximum length there was no shrinkage; transfers carried out after the sections had reached their maximum growth in 1×10^{-3} M IAA showed some shrinkage although not to the same extent as sections growing continuously in this concentration of auxin. This suggests that there is a cumulative effect of the secondary changes during the initial period of rapid elongation.

Experiments carried out to define the relationship between changes in fresh and dry weights and the elongation of sections growing for a long time in optimal

and supra-optimal concentrations of auxin are reported in Table 1. Two sections were obtained from each coleoptile at the age of 90–92 hr and initial fresh and dry weights were determined on a random sample of 50 sections. Similar samples were placed in 1×10^{-5} M and 1×10^{-3} M IAA for the periods of time indicated. The weights include the primary leaves which were not removed from the cylinders in these experiments. The dry weights were obtained after drying the sections for 24 hr at 80°C.

The following differences were observed: (1) elongation in 1×10^{-5} M IAA was closely paralleled by water uptake whereas in 1×10^{-3} M IAA, where the net elongation was very small, there was a considerable loss of water; (2) sections growing in 1×10^{-5} M IAA lost 16 per cent. of their dry weight but sections in 1×10^{-3} M IAA lost 44 per cent.

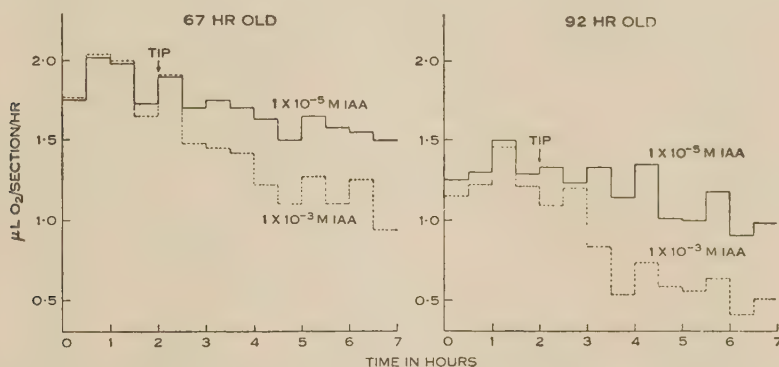


Fig. 5.—Changes in respiratory rates of coleoptile sections (67 and 92 hr old) upon exposure to a high concentration of indoleacetic acid (IAA).

The considerable loss of dry weight in the high concentration may be due to either or both of the following causes: (1) an excessive utilization of metabolites during the initial period of rapid elongation; and (2) a leakage of the cell contents into the bathing solution because of damage to the cytoplasmic membranes, a proposition that is supported by the data on water loss which suggests a mass movement of materials out of the cells. These two possibilities were explored in detail.

(i) *Respiration in the Presence of High Concentrations of Auxin.*—The respiration of *Avena* sections (40 per flask) was measured in standard constant-volume Warburg respirometers at 25°C. Respiration has been expressed as oxygen uptake per section. In a series of preliminary experiments it was established that there was no appreciable difference in the course of elongation or respiration between sections growing in complete darkness or in diffuse light. It is permissible, therefore, to compare elongation data obtained under dark conditions and respiration data obtained in weak diffuse light.

Respiratory rates were measured in sections of two different ages (67 and 92 hr). For the first 2 hr all sections were in 1×10^{-5} M IAA and then the concentration in some flasks was raised to 1×10^{-3} M. The results (Fig. 5) show that exposure to high concentrations of auxin results in a prompt depression of the respiratory

rate. This result makes it clear that the initial rapid elongation of the sections and the loss of dry weight are not associated with increased respiration. Other characteristic features of the results are: (1) in all cases there is a small initial rise of the respiratory rate in 1×10^{-5} M IAA for the first 2 hr of the experiment, then the rate slowly declines. This behaviour is probably due to the wounding of the sections during cutting as previously suggested by Bonner (1949); (2) there is a difference in the magnitude of respiration with age, the rate being greater in the younger sections.

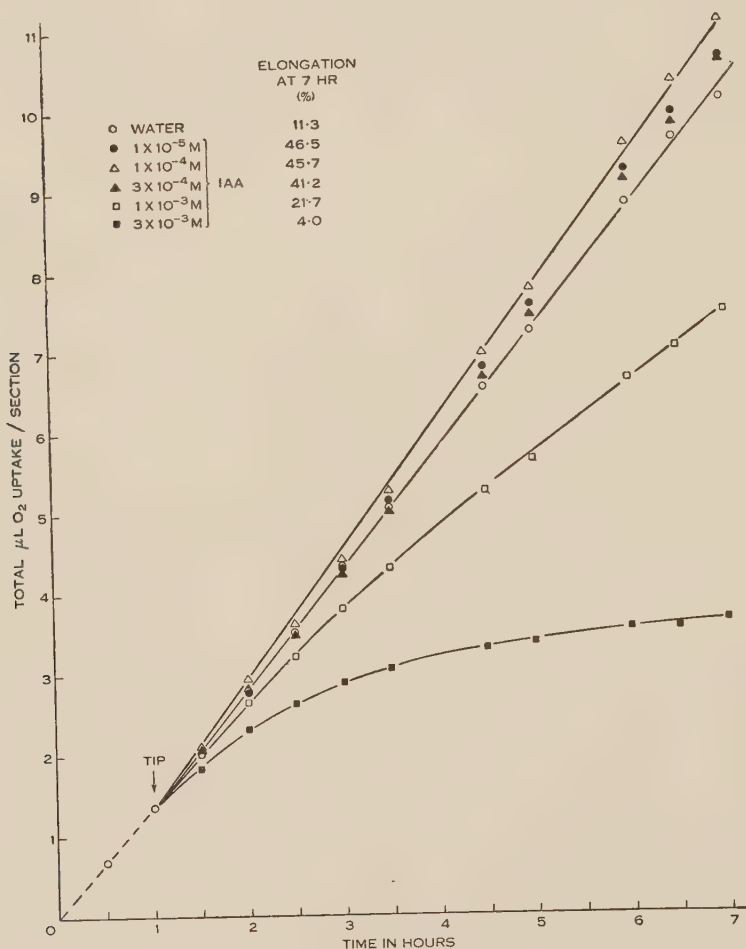


Fig. 6.—Total oxygen uptake and elongation of coleoptile sections over a range of indoleacetic acid (IAA) concentrations.

The lowest concentration at which auxin inhibits respiration was determined in a further experiment in which elongation of the sections was also measured (Fig. 6). It can be seen that inhibition first appears between 3×10^{-4} M and 1×10^{-3} M; moreover, the high concentration effect on elongation also becomes evident within this range. However, there appears to be no quantitative relationship between total oxygen uptake and elongation. For example at the lower auxin

concentrations the total respiration is practically the same as that in water whereas elongation is very different (41–46 per cent. in auxin and 11 per cent. in water).

(ii) *Leakage of Cell Contents Caused by High Concentrations of Auxin.*—To explore the possibility that the loss in dry weight of sections in high auxin concentrations was due to leakage of cell contents, changes in length, fresh, and dry weights of the sections, and recoverable dry weight from the solution were determined at various intervals (Fig. 7). Two sections were obtained from each coleoptile at the age of 96–100 hr and the primary leaves removed from the cylinders. The initial fresh and dry weights were obtained from two replicates of 50 sections each. The remaining sections were then floated in tared watch-glasses containing 5 ml of 1×10^{-5} M or 1×10^{-3} M IAA. Again, two replicates of 50 sections were used in each determination. At the indicated times the sections were removed from their solutions, placed on a glass plate, and then all the liquid from the centres of the cylinders and the plate was recovered with a fine pipette and transferred back into the watch-glass in which the sections had grown. The watch-glass was then placed in an oven and the solution evaporated to dryness (24 hr at 80°C). The sections were dried under the same conditions. The weight of IAA in 5 ml of solution, for the two concentrations used, was subtracted from the dry weights obtained in the watch-glasses. In Figure 7 the changes in length and fresh weight are shown in the upper half of the graph, while the dry weight per 50 sections and the dry weight recovered from the solution are shown in the lower half.

In 1×10^{-5} M IAA elongation and gain in fresh weight follows a similar curve. The lower graph shows a small loss of dry weight (about 15 per cent.) towards the end of the experiment; this is probably due entirely to respiration since there is no indication of leakage. In 1×10^{-3} M IAA the picture is quite different. Water uptake lags behind the increase in length and actually stops before the sections reach maximum elongation prior to shrinking (this characteristic will be considered in a later paper). Then, water is lost at a rate much greater than the rate of shrinkage of the sections. Consequently after 22 hr in 1×10^{-3} M IAA sections had lost 10 per cent. of their initial water content while in the same time they had elongated by about 5 per cent. The rate of loss of dry weight was not constant with time but was maximal between the second and sixth hour from the beginning of the experiment. In all determinations the amount of dry weight lost was recovered from the solution so that the dry weight of the sections plus the dry weight recovered was constant throughout the experiment and equal to the initial dry weight of the sections. It will be noticed that the percentage increases in length and fresh weight in 1×10^{-5} M IAA follow each other approximately. Variations are probably due to the fact that, whereas no two samples had exactly the same fresh weight initially, the percentage increases are based on one initial value since it was impracticable to determine the initial lengths and fresh weights of all samples.

From the above data it appeared that once dry matter starts leaking out of the cells the rate of loss is quite rapid. However, no indication is given if leakage starts immediately upon placing the sections in the auxin solution, or later on, concurrently with the movement of water out of the cells. Table 2 summarizes the results of determinations similar to those described above but limited to the high

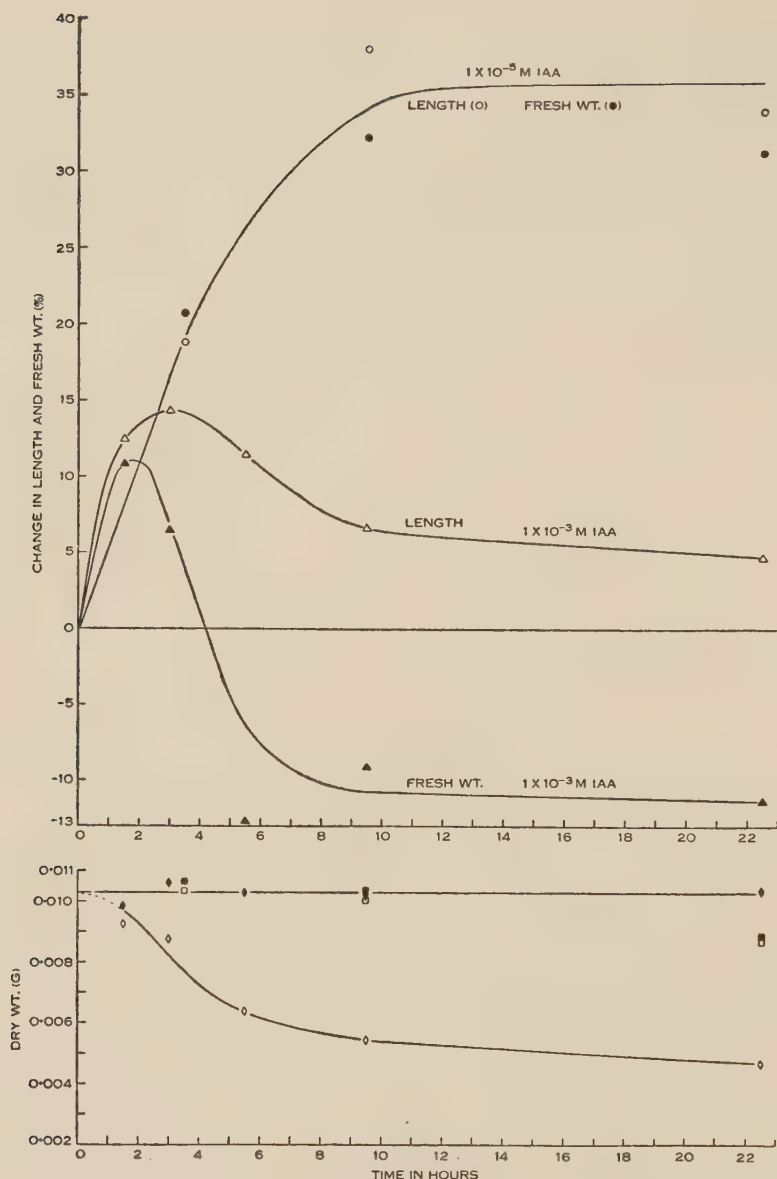


Fig. 7.—Course of the changes in length, fresh, and dry weight of coleoptile sections, and of the material leaking into the solution over 23 hr for two concentrations of auxin. N.B. Lower graphs: □ Dry weight/50 sections in 1×10^{-5} M IAA. ■ Dry weight/50 sections in 1×10^{-5} M IAA plus dry weight recovered in solution. ◇ Dry weight/50 sections in 1×10^{-3} M IAA. ◆ Dry weight/50 sections in 1×10^{-3} M IAA plus dry weight recovered in solution.

concentration of auxin and to the first 2 hr of exposure of the sections to it. For the first $1\frac{1}{2}$ –2 hr of exposure to a high concentration of auxin there was practically no loss of dry weight. Then, at about the second hour and before the sections

started shrinking, water began moving out of the cells together with solid constituents.

TABLE 2

CHANGES IN LENGTH AND IN FRESH AND DRY WEIGHTS OF COLEOPTILE SECTIONS, AND OF MATERIAL LEAKING INTO THE SOLUTION OVER THE FIRST 2 HR OF EXPOSURE TO $1 \times 10^{-3}M$ INDOLEACETIC ACID

All weights are expressed as grams per 40 sections

Time (min)	% Increase		Section Dry Wt. (g)	Recovered Dry Wt. (g)	Section Dry Wt. plus Recovered Dry Wt. (g)
	Length (micrometer units)	Fresh Wt. (g)			
0	—	—	0.0074	—	—
35	2.0	1.1	0.0066	0.0007	0.0073
80	3.4	1.5	0.0067	0.0008	0.0075
125	6.3	4.5	0.0063	0.0010	0.0073

IV. DISCUSSION

The most notable feature of this work is the increase in the initial elongation rate of coleoptile sections with increasing concentrations of auxin, even within the range of concentrations normally considered inhibitory to growth. In these high concentrations the initial rapid elongation is followed by shrinkage and death of the sections. As described in the Introduction, these results have a bearing on certain concepts which attempt to explain the mechanism of auxin action in cell elongation. For example, two aspects of the problem that need to be reconsidered in the light of the presented results are the mechanism of growth suppression by supra-optimal concentrations (Foster, McRae, and Bonner 1952) and the suggested application of the methods of enzyme kinetics in the analysis of growth induced by auxins (Bonner and co-workers).

The experiments in which the respiration of coleoptile sections was compared with their growth in different concentrations of auxin showed that the initial stimulation of elongation by high concentrations was not accompanied by an increase in respiration (Fig. 5); on the contrary, there was a considerable drop of oxygen uptake during that period. This emphasizes the lack of any stoichiometric relationship between elongation and respiration as demonstrated in the optimal range of auxin concentrations (Berger, Smith, and Avery 1946) and also in the course of this work (Fig. 6). Therefore, whatever the mechanism of cell expansion may be, any dependence of the process on respiration must be an indirect one. Since the synthetic reactions which are associated with growth require the expenditure of energy it is obvious that they can take place over an extended period of time only if there is continuous respiratory activity to provide that energy; the depression of respiration must eventually have an inhibiting effect on such reactions. Energy stored in some

form may be sufficient to meet the requirements of the rapidly elongating cells for the first 2 or 3 hr of growth in 1×10^{-3} M IAA. Depletion of the energy reserves, plus the fact that they are not restored, because of the low respiratory activity of the tissues, may be contributing to events that bring about the cessation of growth and the subsequent death of the sections.

Another characteristic of the behaviour of coleoptile sections in high concentrations of auxin is that the shape of the elongation curve changes with the age of the tissues. There is a tendency for the effects of the high concentrations to diminish as the sections are obtained from younger plants. The initial rate of elongation is reduced and at the same time the peak is reached after a longer exposure to auxin. These points suggest that younger tissues are less susceptible to the injurious effects of the higher concentrations than older ones. As the cells age, changes must take place in the structure of the walls and the enzymic constitution of the cell which determines the reaction of the tissue to a given set of conditions (Brown, Reith, and Robinson 1952).

The observed loss of water and dry weight from the sections that characterizes the cessation of growth and shrinkage in high concentrations of auxin (Fig. 7, Tables 1 and 2) cannot be attributed to a rise in respiratory activity of the tissues. However, there is evidence (Kelly 1947) that reduced levels of respiration, whether caused by respiratory inhibitors or by low oxygen tensions in the environment, may result in the loss of cell contents. Bennet-Clark and Kefford (1954) commenting on the possible nature of the early decrease in growth rate in the higher concentrations of auxin are of the opinion that it is due to either an accumulation of inhibitors inside the sections or to injury of the protoplasm. The experiments described above suggest that injury and destruction of the protoplasm and its membranes are probably the cause of the cessation of growth and the subsequent shrinkage of the sections.

Cell expansion is essentially a process of increase in area of the cell wall and a promising theory on the mechanism responsible, put forward by Frey-Wyssling (1950, 1952), proposes that the whole process can be reduced, broadly, to two sets of reactions. Firstly, those which are synthetic in nature and tend to build up the cell wall, and secondly, reactions which tend to dissolve the cellulose framework by reducing the strength of the bonding between the micelles. The relative intensities of the two sets of reactions determine whether growth and consolidation or dissolution of the cell wall is to take place. For wall expansion to occur there must be a balance between the two; dissolution reactions are necessary to keep the cellulose framework mobile and at the same time the building-up processes must produce new wall material. Under normal conditions synthetic reactions may predominate. These reactions are intimately linked with protoplasmic activity since the primary wall of an expanding cell is an integral part of the protoplasm. If the dissolution reactions predominate expansion will eventually cease and finally the protoplasmic structure will be disrupted as well.

The results presented in this paper suggest that such a scheme is operating during auxin-induced growth in coleoptile sections. It appears that for a given increase in auxin concentration both synthetic and dissolution reactions are accel-

erated but that there is a relatively greater increase in the rate of the dissolution reactions. This two-process scheme explains how with an increasing concentration of auxin the initial rate of elongation is greater due to the greater "mobility" of the cellulose structure, the duration of growth is shorter, and finally, the breakdown of the protoplasmic structure is brought about with the ensuing loss of water and solid materials from the cells.

The results of this work raise some queries regarding two concepts on the action of auxins. The first concerns the "two-point attachment" theory developed by Muir, Hansch, and Gallup (1949) and Muir and Hansch (1951), and the second the application of enzyme kinetics to auxin-induced growth. On the basis of the "two-point attachment" theory, Foster, McRae, and Bonner (1952) have suggested that the inhibition of elongation brought about by supra-optimal concentrations of auxin—it must be remembered that it is the long-term effect of high concentrations that is considered—is the result of a competition between auxin molecules for the available sites in the cell.

The above explanation of inhibition does not satisfy the data reported here. It has been shown that the initial effect of supra-optimal concentrations is an acceleration and not an inhibition of elongation although secondary damaging effects tend to obscure the true picture. Very similar observations have already been reported by Bennet-Clark and Kefford (1954). This, of course, does not imply that the "two-point attachment" theory is invalid. It merely suggests that a competition for the reactive sites within the cell is not the complete picture of the events in the higher concentrations, up to $1 \times 10^{-3}M$, otherwise the initial rapid rates of elongation would not materialize.

The second point that is raised by these experiments concerns the application by Bonner and his group (Foster, McRae, and Bonner 1952; McRae and Bonner 1952; McRae, Foster, and Bonner 1953) of the methods of enzyme kinetics in the analysis of auxin-induced growth. In this treatment the entire coleoptile section is treated as a one-enzyme system and a number of assumptions are made. It is, for example, assumed that the auxin becomes bound to some receptive site within the cell, in the manner outlined previously, and that a ready equilibrium is established between the free and bound forms. The initial rate of the resultant reactions, i.e. of elongation, which must be known accurately for the application of the methods, is taken to be proportional to the concentration of the bound auxin. Also, it is assumed that the concentration of the receptive sites is small by comparison with the concentration of the auxin molecules. The standardized conditions used by the above workers included a basal medium of 3 per cent. sucrose and 0.0025M potassium maleate buffer (pH 4.5) to which the auxins were added. The initial elongation rates of the sections were obtained from a single reading after 12 hr of growth and it is claimed that elongation during that period is linear. In view of the experiments reported here this method needs to be reconsidered. The treatment of the coleoptile as a one-enzyme system with constant properties has been criticized already. Although auxin-induced growth may be associated with a single enzyme, in the sense that the auxin molecule becomes bound to a definite receptive entity in the cell, there are many reactions associated with other enzymes which must

also influence growth. On this basis it cannot be assumed that a coleoptile section remains a system of constant properties during its growth over a period of many hours. It follows, therefore, that a treatment such as the one outlined above may lead to misinterpretation of data.

Bennet-Clark and Kefford (1954) also claim that the relationship between section growth and time is not linear over a range of auxin concentrations and for a period of over 12 hr. Bonner and Foster (1955) point out that the failure to obtain straight-line relationships between growth and time for a period of at least 12 hr is due to the use of unsuitable experimental conditions: (1) Bennet-Clark and Kefford used plants younger than those recommended. This criticism does not apply to the experiments reported in this paper where the plants used were about 90 hr old, i.e. slightly older than Bonner's plants (84 hr). (2) Another criticism, levelled against the use of unbuffered auxin solutions, is also applicable to the experiments reported here. It is, however, unlikely that the lack of pH control would influence the results in the manner claimed: "The use of unneutralized auxin solutions and the absence of buffer permits the higher auxin concentrations to cause irreversible damage to the sections merely by acidity." Coleoptile sections in optimal concentrations of auxin can grow normally in solutions of pH as low as 4.0 (Bonner 1934) and any effect of pH on growth is largely due to its effect on the growth substance itself. In the more acid medium more of the substance is found in the non-dissociated, active form. Also, it must be remembered that in $1 \times 10^{-3}M$ IAA, with a pH of 4.0, the initial growth of the sections is greater than that in optimal concentrations. If the acidity of the medium is harmful then why the initial period of rapid growth? (3) The third difference in experimental conditions is the absence of potassium ions which have been shown to have an effect in maintaining the growth rate of coleoptile sections, particularly after the first few hours of growth (Thimann and Schneider 1938). In this connection Bennet-Clark and Kefford (1954) refer to unpublished data by H. M. Hurst on the effect of potassium and sodium ions on the elongation of coleoptile sections and conclude: "In the presence of ions the initial rapid extension rates brought about by auxins are considerably reduced and may thus have passed unnoticed by the Californian workers." In view of these effects of potassium ions it is not absolutely certain whether the measured response of the sections is due to the concentration of auxin molecules, the potassium ions, or to a combination of the two.

The conclusion that one can draw by comparing the reports of Bonner and his group, on one hand, and of Bennet-Clark and Kefford and of the author, on the other, is that the application of the methods of enzyme kinetics in the analysis of growth of coleoptile sections in auxin solutions must be approached with great caution. Apparently the linear response with time can be realized only under strictly limited growth conditions and in the presence of additives, like potassium ions, which may complicate the response of the tissue. Small deviations from these specified conditions can lead to completely different conclusions regarding the nature of auxin action. This point is illustrated in this paper by the increasing initial rate of elongation with increasing auxin concentration and the doubt that this situation casts on the suggestion that "growth inhibition" in high concentrations

of auxin is due to a competition by the auxin molecules for available sites on a reactive entity in the cell.

V. ACKNOWLEDGMENTS

The author wishes to express his thanks to Professor F. L. Milthorpe for suggestions in the course of the work and to Dr. L. H. May for his helpful criticism during the preparation of this paper.

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ELECTRIC OSCILLATIONS GENERATED BY PLANT ROOTS AND A POSSIBLE FEEDBACK MECHANISM RESPONSIBLE FOR THEM

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[*Manuscript received October 30, 1956*]

Summary

An account is given of rhythmic variations which have been observed in the electric field generated by bean roots growing in water. These electric oscillations which have periods of about 5 min have been studied using apparatus which records automatically at frequent intervals the potential pattern near the root. A description is given of this apparatus and of a very sensitive instrument for recording the rate of elongation of the root.

The cause of these and similar oscillatory phenomena in biological systems is discussed and a possible feedback mechanism responsible for the observed electric oscillations is postulated.

I. INTRODUCTION

In an earlier paper (Scott, McAulay, and Jeyes 1955) a new method of measuring the bioelectric fields generated by plant roots was described. A root was grown vertically in a bath of water made weakly conducting with KCl. Potential differences were then observed between points in the water adjacent to the root due to electric currents generated by the root and flowing through the water. This method was claimed to be an improvement on those employed in previous investigations. Interference with the normal growth of the root by the measuring process was avoided, leading to results which appeared to be more consistent than those reported previously.

In the earlier paper the steady electric pattern generated by a normal, growing root was described. A more detailed study of this pattern has shown that under some circumstances large periodic fluctuations in the electric potentials are generated (McAulay and Scott 1954). These oscillations are superimposed on the steady or background pattern. They are sinusoidal in form and have periods of the order of 5 min, amplitudes of a few millivolts, and may persist for several hours. The observed oscillatory pattern and the conditions under which it is generated will be described in detail in Section III.

Study of these variations in electric pattern has been made practicable by the construction in this Laboratory of apparatus which automatically measures and records at frequent intervals electric potential differences between a number of points in the bath adjacent to the root. This apparatus will be described in Section II.

A new apparatus for measuring and recording the rate of elongation of a root simultaneously with the measurement of its electric potential pattern is also described in Section II. This apparatus was constructed in order to discover whether rhythmic oscillations of rate of growth accompanied the electric oscillations. Observations made with this equipment are described in Section III(d).

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The observation of rhythmic changes in the electric field of roots has raised the general question of the cause of these and similar oscillatory phenomena associated with biological material which do not appear to be related to any corresponding oscillatory changes in the environment. Possible mechanisms for the generation of such oscillations will be discussed in Section IV and in particular a possible source of the electric oscillations described in this paper will be suggested.

II. APPARATUS AND MATERIALS

(a) *Automatic Apparatus for Measuring and Recording Potential Differences between a Number of Points near Plant Roots Grown in Water*

The apparatus used for automatically recording bioelectric potential differences near bean roots growing in weakly-conducting water is shown in Figure 1. A

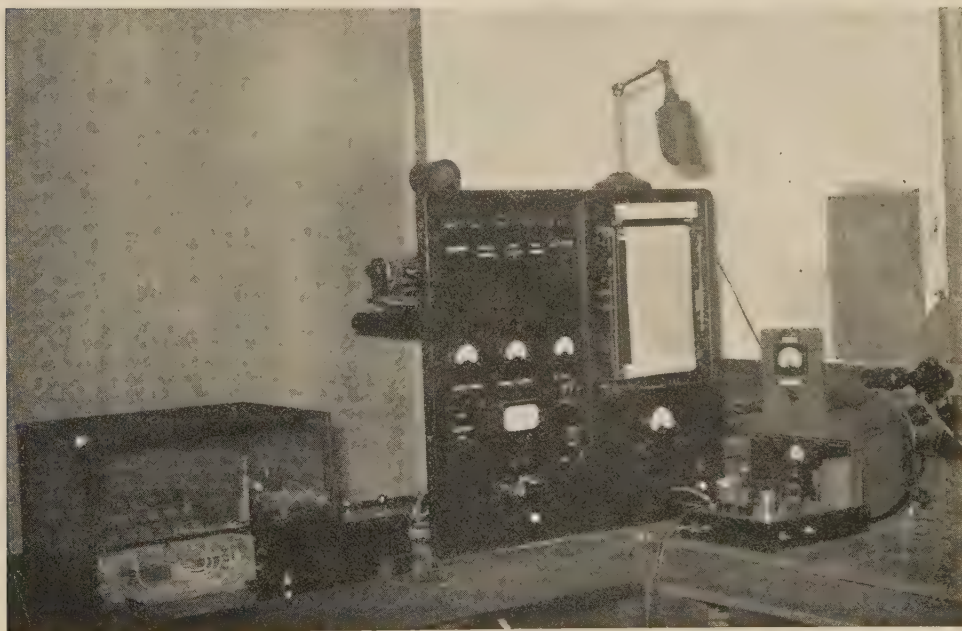


Fig. 1.—Apparatus for making automatic records of plant potential differences.

schematic diagram is given in Figure 2. The basic recording instrument used was a Cambridge six-channel, quick-acting recorder with a maximum full-scale deflection of 1 mV. This recorder produced dots of different colours to distinguish the different channels. The time between successive dots was 5 sec so that each channel was recorded at $\frac{1}{2}$ -min intervals. For most of the work described in this paper the chart was driven at 1.5 in./hr.

The recorder had to be modified so that it could be used with an electrometer stage input. This was necessary because the input resistance of the recorder was only 1000Ω , whereas an input resistance of the order of $10^{11}\Omega$ was required for the bioelectric measurements to be described in order to prevent undue current drain

and resulting polarization of the source. The electrometer used a pair of matched ME 1400 valves in a balanced circuit and was designed to be as insensitive as possible to changes in H.T. or L.T. voltage. Currents flowing in the input circuit of this stage were never more than 10^{-12} A under the conditions of measurement.

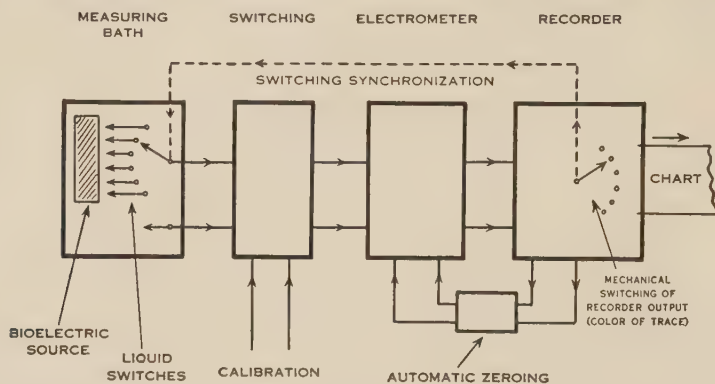


Fig. 2.—Schematic diagram of the automatic apparatus for measuring plant potential differences.

A photograph of the measuring bath is shown in Plate 1. It was constructed of "Perspex" and had the dimensions $11 \times 5 \times 3$ in. It was filled with 0.0001N KCl solution which was aerated, stirred, and temperature-controlled. For most of the measurements described in this paper the temperature was controlled at 25°C , although one or two experiments were conducted at temperatures as low as 17°C .

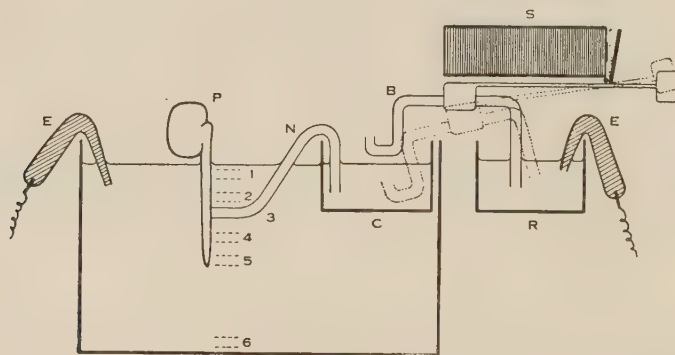


Fig. 3.—Section through the measuring bath to illustrate the liquid-switching arrangement. For simplicity only one of the liquid-conducting paths from the plant to the rear bath is shown.

The plant under investigation *P* was mounted in the tank with the root immersed vertically (Fig. 3). The ends of lengths of transparent "Nylex" tubing *N* (3-mm bore) were brought close to the surface of the root at various points along its length. The other end of each tube dipped into an insulated plastic cup *C*, the cups and tubes being also filled with 0.0001N KCl. Tubes came from as many as

five points near the plant. A sixth tube came from a point in the bath some distance from the plant; this was used as a reference (see later).

Each of these cups was connected in turn to the measuring circuit by means of a liquid-switching arrangement. Associated with each cup was a glass bridging-tube *B* (also filled with the same KCl solution) which could dip into *C* and make a connection across to a small 0.0001N KCl bath *R* at the rear of the main bath and insulated from it. The bridging-tube made this connection when the solenoid *S*, on which it was mounted, was energized. The six solenoids were energized in succession, synchronizing with the recorder output channels (colour of trace on chart). One of the electrodes *E* (calomel half-cells) dipped into the rear bath, and the other into the main bath at a point some distance from the plant. These electrodes were connected to the electrometer. Thus it is seen that each point near the plant was connected in turn to the rear electrode via a path which was entirely of 0.0001N KCl.

Water was nowhere allowed to become stagnant. A fresh supply dripped into the rear bath and passed through the bridging and "Nylex" tubes to the main bath where the excess dripped out at an overflow.

It might appear that the rather complicated bath-switching arrangement described above and which required high insulation could have been avoided if, instead of using a single pair of electrodes and a single electrometer, separate electrometer stages and electrodes were used for each channel. Selection of the various channels for measurement could then be done by a relatively simple metallic selector switch in the recorder itself.

The former system was used, partly to avoid duplication, but more especially because electrometer circuits and half-cells are never absolutely stable, but show slow potential drifts over long periods of time. In order to determine whether an observed change in potential is a genuine plant effect or due merely to a drift in the measuring apparatus, it is necessary to "switch the plant out", i.e. to measure the potential at a point in the bath remote from the plant and where the potential is presumably unaffected. To obtain a reference reading with an automatic recorder it is therefore necessary either to move the measuring probe away from the plant to a distant point at frequent intervals, or to use some sort of bath switch such as that described above, and for one channel to switch to a distant point.

In the present case the reference potential was recorded at 30-sec intervals. For convenience in reading the charts any drifts in the measuring apparatus were automatically compensated for, so that the reference trace always showed no deflection from zero on the chart. A phase-sensitive motor which drove the zeroing control in the electrometer was energized through contacts in the recorder whenever the reference potential differed from zero.

(b) Growth Meter which Automatically Records the Rate of Elongation of Plant Roots Growing in Water

It was desired to find a sensitive method of measuring the rate of elongation of a root in order to test whether rhythmic variations occurred in this rate and

if so whether they correlated with the electric rhythms detected with the recording apparatus (Section II(a)) and described in detail in Section III.

The rate of growth of a moderately active root is about $10\ \mu/\text{min}$. Consequently in order to observe small oscillatory variations in this rate, the periods of which might be only a few minutes, it was desirable that increases in length of about $1\ \mu$ should be detected. In addition to this requirement the method had to be suitable for use with roots growing in water, it should not interfere with the potential measuring apparatus, and it should lend itself readily to automatic recording of the growth rate.

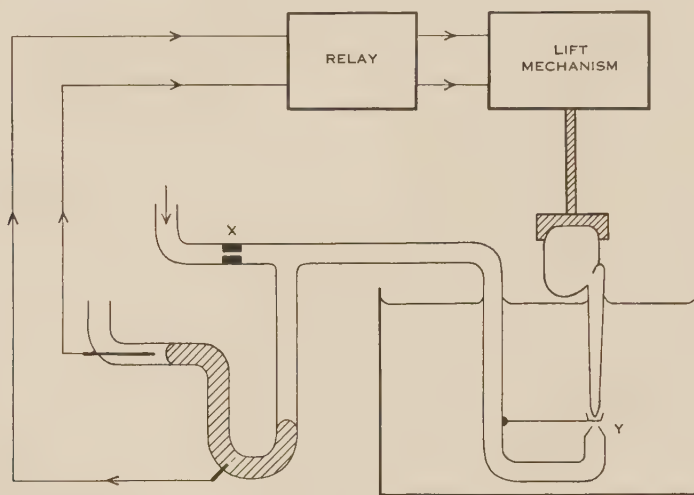


Fig. 4.—Simplified diagram illustrating the principle of the rate-of-growth meter.

None of the conventional methods appeared to be suitable. Optical methods could not easily be applied. Auxanometers of the type described by Koningsberger (1922) and recently modified by Ranson and Harrison (1955) could not be used because of electric interaction and the difficulty of keeping the switch contacts dry.

The method which was finally chosen was suggested by Dr. D. B. Idle of the University of Birmingham (personal communication). The apparatus is shown diagrammatically in Figure 4. KCl solution ($0.0001N$) from a constant head (about 120 cm of water) flowed along a tube in which there were two constrictions X and Y which restricted the flow of water by about the same amount. The constriction at Y was variable being covered by a flexible flap against which the root tip pressed lightly.

The method made use of the fact that a small increase in the resistance to the flow of water at Y caused a considerable increase in the water pressure between X and Y. This increase caused electric contacts in the mercury manometer to close, and the lift mechanism was activated. The plant was then raised through a small increment ($1\ \mu$) thereby reducing the pressure on the flap and breaking the electric contact in the manometer. Thus the growing plant was raised up in $1\ \mu$

steps each time the manometer contacts were closed. During an active period of growth the plant was raised by $1\ \mu$ increments at intervals of 2 or 3 sec.

The lift mechanism employed the screw and ratchet of a Cambridge rocking-arm microtome and this was operated by means of a solenoid. Power to energize the solenoid came from a relay circuit as an electric pulse each time the manometer contacts closed. In the event that a single upward step by the plant was insufficient to break the contacts (as would be the case, for example, if the root grew several microns suddenly) it was arranged that pulses from the relay should continue to energize the solenoid at 1- or 2-sec intervals until the contact broke. The number of increments was counted on a mechanical register.

In order to facilitate comparison of the electric pattern with the rate of growth, it was decided to record the number of growth increments each minute on the same chart as was used for the potential difference measurements. Each pulse from the relay turned the movable contact of a variable resistance through a small angle. Because a constant current was passed through this resistance the potential difference across it was proportional to the number of increments. This potential difference was registered by the recorder each minute, one of the channels being used for the purpose. The integrator was then automatically reset to zero to count the number of increments during the next minute.

(c) *Material*

The material used in the experiments described in this paper was the broad bean, *Vicia faba* L., var. Johnson's Long Pod, which was grown in water-baths at 25°C. In most cases tap water was used, but in some the growth medium was 0.0001N KCl. The water was circulated and aerated. Plants 2-3 days old with roots about 30 cm long were used in most experiments.

III. RESULTS

(a) *Root as Source of Electric Oscillations*

Whenever a new phenomenon is being studied it is especially important to check whether all possible artifacts have been accounted for and removed. In the present case careful tests were made to eliminate processes other than those due to the plant which could have given rise to the observed electric oscillations.

Such oscillations clearly could not have arisen in the electrodes, electrometer, or recorder, as the reference trace giving the potential at a point remote from the root in the bath showed no oscillations. Since the temperature of the measuring bath was thermostatically controlled it was necessary to check whether the electric oscillations correlated with the switching off and on of the heater. No such correlation was found; in fact, the electric oscillations still took place when the thermostat was disconnected. In a similar way it was shown that small fluctuations of height of the water in the bath were not responsible for electric fluctuations. Interaction with neighbouring electrical circuits was also ruled out.

That the root was itself the source of the electric oscillations could be seen most conclusively by moving the measuring probes in the vicinity of the root. The

amplitude of the oscillations became smaller as the probes were moved away from the root and varied with the position of the probes along and around the root. It is therefore concluded that the electric oscillations were generated by the plant itself.

(b) *Description of the Oscillatory Pattern*

The results obtained in 26 cases of bean roots which were observed to generate regular and persistent electric oscillations have been analysed. The general features of this oscillatory pattern will now be described.

When a plant was taken from the growth bath to the measuring bath and allowed to settle for some time (about 1 hr), the electric pattern in the water surrounding the root usually became steady, although slow changes occurred over long periods of time. This steady pattern agreed with that described previously (Scott *et al.* 1955); the region in the vicinity of the root tip being more negative than the root base. This indicated that an electric current flowed out from the plant at the root base, entering it again in the vicinity of the tip.

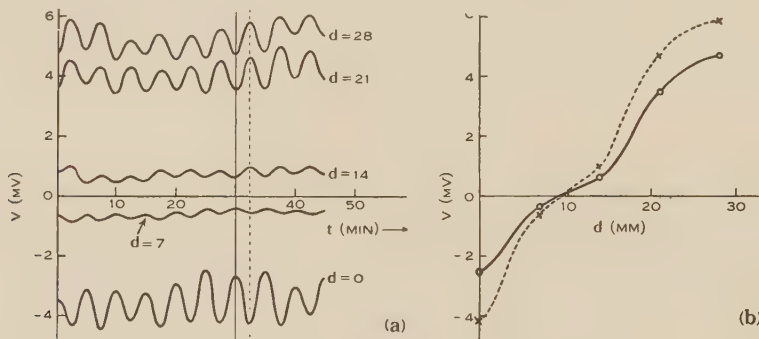


Fig. 5.—(a) Electric oscillations generated by a bean root. Each graph shows the time variation of the potential V (measured relative to a distant point in the bath) at a point close to the root a distance d mm from the tip. (b) Corresponding graph of V against d . The two curves are the extreme forms of the standing-wave pattern a half cycle apart. Note the phase reversal about the node 9 mm from the tip.

In the case of the group of plants now under consideration, oscillatory variations were observed to be superimposed on this normal steady potential pattern. These oscillations commenced from 1 to 10 hr (with an average of 4.7 hr) after setting the root up for measurement, and continued for an average of 5.8 hr. The average number of oscillations generated by the plant during this time was 55.8.

It was noticeable that the oscillations were very nearly sinusoidal in form. Certainly they never resembled relaxation oscillations. For a particular plant the period of oscillation at all points in its vicinity was the same and remained remarkably constant while the plant was in the oscillatory state. For a typical root, the average period for 42 cycles was 5.68 min with a standard deviation of 0.60 min. For the majority of plants studied the period was of this order, although in four cases it was longer, ranging from 12 to 32 min. If these slowly oscillating cases are excluded, the mean period for the main group was 5.75 min with a standard deviation of 1.02 min.

The amplitude of oscillation varied with position along the root. The most active region was nearly always within 8 mm of the tip. The average over a number of plants for the maximum peak-to-peak amplitude of the oscillation in the most active region was 1.7 mV although in two cases amplitudes as large as 5 mV were recorded. Although the amplitude of oscillation at a particular point usually remained approximately constant over many cycles, it was observed in five cases that the amplitude slowly built up to a maximum value, then fell away to zero, the process being repeated several times. The period of these "beats" was about 2 hr.

A study of the phase relationships of the oscillations taking place at several points along the root has revealed some interesting data. In most cases the oscillatory pattern was in the form of a standing wave, the oscillations taking place above and below a particular point (node) along the root being reversed in phase (Fig. 5). In some cases two nodes were observed, the oscillations near the tip and the root base being in phase, but opposite in phase to those of a region in between (Fig. 6).

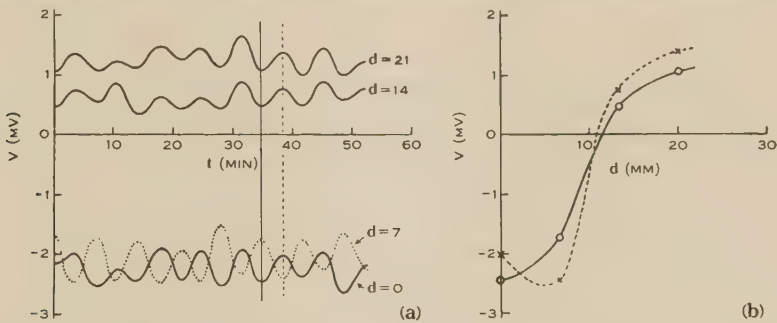


Fig. 6.—As for Figure 5, except in this case the standing-wave pattern has two nodes.

Such phase reversals are to be expected, since a rise in potential of the water near one part of the root must be accompanied by a fall somewhere else along the root, if the electric current leaving the root is always to balance exactly the current entering it. As large oscillations were often observed near only a relatively small part of the root it appeared that the active source of the electric oscillations in these cases was confined to this region. Other parts of the root were inactive, small oscillations being observed there merely because of the return path of the oscillatory current through them.

In a few cases (Fig. 7) the phase relationship was considerably more complicated suggesting that a wave of activity was moving along the plant. It is seen that the oscillations were in phase near the root base, but closer than 15 mm to the tip the phase changed with position and was not related in a simple manner to that at the root base.

A pattern of this type might be produced by a root having more than one active source. If this were so, some mechanism would be required to synchronize the sources to oscillate at very nearly the same period, as the phase differences were found to remain almost constant for more than 30 cycles. As suggested above a more plausible explanation is that a disturbance generated by a source moved in

the form of a wave along the root thus causing electric oscillations which lag more and more in phase as the distance from the source is increased. In the present example, the observed phase relationship would be consistent with that caused by a wave travelling up the root from the tip with velocity 3.0 mm/min or alternatively down the root with velocity 1.4 mm/min. The phase relationships warrant further study, particularly to test whether the phase changes continuously along the root in the active region.

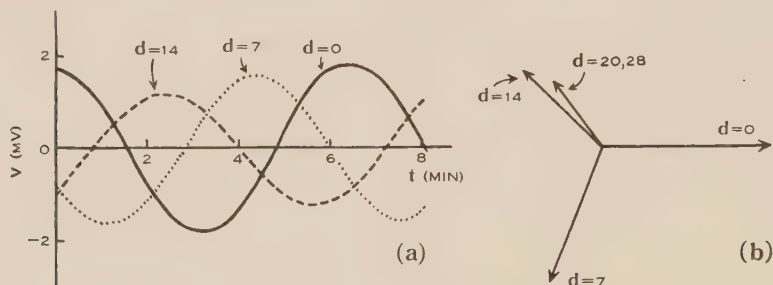


Fig. 7.—Illustration of the complex phase relationship sometimes observed. (a) The time variation of the potential V at points along the root d mm from the tip. For simplicity only the oscillatory component of V is shown. (b) A vector diagram illustrating the amplitude and phase relationship of the oscillations taking place for various values of d .

(c) *Transient Oscillations Resulting from Stimulation*

The electric pattern of a bean root was very sensitive to stimulation and changes in environment, large changes in the electric potential along the root being produced immediately. When the cause of the disturbance was removed, the pattern again became practically steady after a time which might range from a few minutes to an hour, depending on the degree of stimulation.

During this transitory time before the steady pattern is restored, the potential changes were frequently in the form of damped oscillations (Fig. 8). Such oscillations almost always occurred when a plant was taken from the tank in which it was grown and set up for measurement. Overshoot processes followed by damped oscillations were also observed to result from such treatments as mechanical stimulation of part of the root, sudden changes in the salt content of the water in the bath, addition of indoleacetic acid to the bath, and exposure of the root to air for a short time.

In a preliminary study of these patterns it was found that the period of the transient oscillations was of the same order as the maintained oscillations described in Section III(b) although the variability was greater, both during the oscillation of one plant; and from plant to plant. Further study of these transient electric changes is planned.

(d) *Observations of the Rate of Elongation of the Root*

Since an earlier paper (Scott *et al.* 1955) had shown some correlation between the steady electric pattern and the mean rate of elongation of the root, it was

considered possible that the observed oscillatory electric changes might be accompanied by corresponding oscillations in the rate of elongation.

The rate of elongation of the root was measured and recorded simultaneously with the electric potential measurements, using the apparatus described in Section II. No special tests were made of the accuracy and reliability of the growth meter, but this was considered to be good judging by the small amount of scatter (less than 10 per cent.) which was observed in the rate-of-growth record of some roots. Other roots showed more random scatter than this, and this was considered to be due to a genuine variability in the rate of growth, although no exhaustive tests have yet been made of other possible sources of scatter.

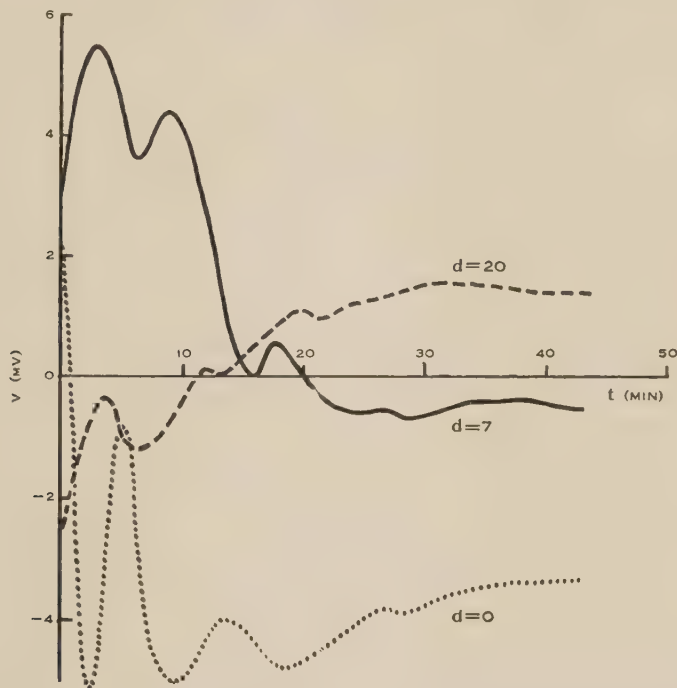


Fig. 8.—Damped oscillations in the potential pattern following stimulation. The symbols have the same meanings as in previous graphs.

On a few occasions, variations which were approximately periodic were observed (Fig. 9) but these did not occur while electric oscillations were being generated and there appeared to be no correlation between the two. It is, of course, possible that growth oscillations too small to be detected were taking place during the time of electric oscillation. If this were so, the amplitude of the oscillations must have been less than about 15 per cent. of the mean growth rate in order to escape detection.

The lateral movements of bean roots have been studied using time-lapse photography. It has been found that the growing root usually spirals around a vertical axis with a period of about 2 hr. So far there is no indication of a 5-min rhythm superimposed on this.

(e) Correlation with Pressure Applied to the Root Tip

A large number of treatments have been applied to a root producing its normal steady electric pattern with a view to causing it to start generating electric oscillations. None of these treatments has been entirely satisfactory. Nevertheless there is some evidence that pressure applied to the root tip tending to prevent elongation is one important factor required for the oscillatory state.

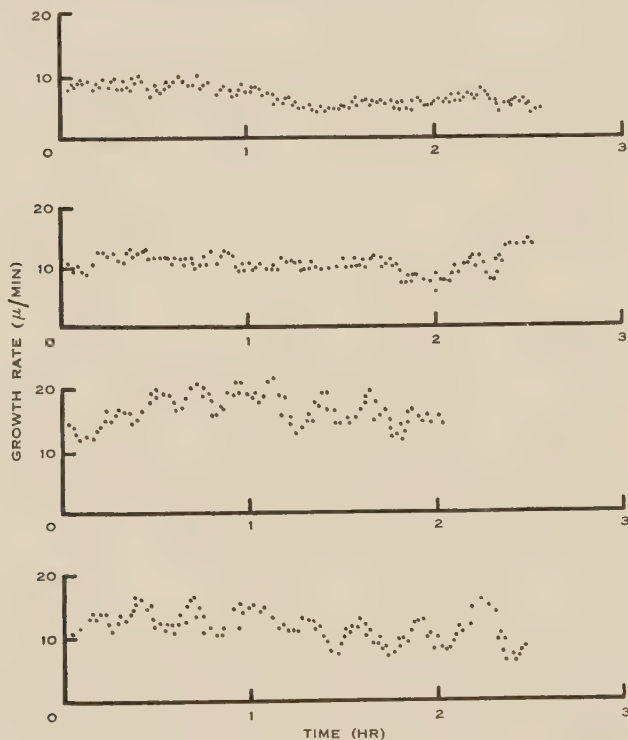


Fig. 9.—Tracings from four charts showing the rate of growth of bean roots as measured and recorded by the apparatus described in Section II. Note the presence of oscillations in the rate of growth in the two lower graphs. These were not found while electric oscillations were being generated by the root. The period of the growth oscillations is rather longer than most of the electric oscillations.

On only two occasions were roots which were not experiencing tip pressure observed to be generating electric oscillations. On 18 other occasions, oscillations were in each case found to be associated with the presence of an upward force on the root tip. In some cases oscillations were produced when the root grew against a fixed obstacle, while in others they were produced when the root tip experienced a constant upward force of about 100 dyne. These oscillations usually commenced soon after pressure was applied to the tip, and lasted for some time after it was removed, although in one or two cases oscillations ceased while the root was still experiencing pressure. Permanent bending of the root frequently resulted from these tip pressures.

Although the above results suggest that tip pressure is an important contributing factor for the oscillatory state it is not true to say that oscillations always arise if tip pressure is applied. On a large number of occasions roots were subjected to tip pressure and no electric oscillations were observed.

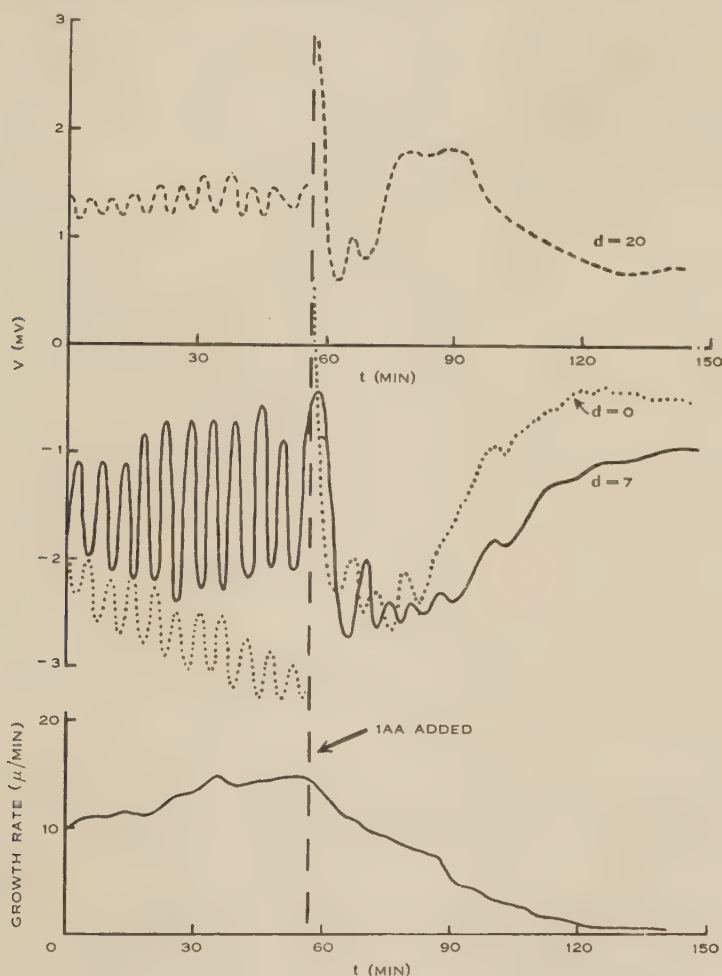


Fig. 10.—Effect of indoleacetic acid (IAA) on the potential pattern and rate of growth of the root. IAA (2 mg/l) was added to the bath containing a root generating electric oscillations at a time indicated by thick broken line. The symbols have the same meaning as in previous graphs.

These preliminary investigations suggest that the plant must comply with certain other conditions, as yet not ascertained, before it has the capacity to generate electric oscillations. Only then will it oscillate when pressure is applied to the tip.

(f) *Effect of Auxin*

In one series of experiments indoleacetic acid (IAA) was added to the measuring bath in which a root was generating electric oscillations. The concentration of IAA

was usually made 2 mg/l. At this strength the auxin inhibits elongation of the root (Scott *et al.* 1955).

In each case it was observed that IAA had a marked effect on the electric oscillations. A typical example is shown in Figure 10. It will be seen that the electric potentials were immediately altered (stimulation effect) after which the oscillation was rapidly damped. At the same time the rate of growth fell until the root had practically stopped elongating about 1 hr after the addition of auxin. After several hours the plant was sometimes observed to generate an oscillation again and at about the same time the root in some cases resumed elongating slowly. As the experiment was performed in daylight it was thought possible that the auxin effect was reduced due to destruction of the auxin by light.

IV. DISCUSSION

Rhythmic oscillatory processes are frequently observed in living systems. Most of these rhythms are clearly related to rhythmic changes in the environment such as diurnal, annual, or tidal changes or periodic variations in light or temperature (Kleitman 1949).

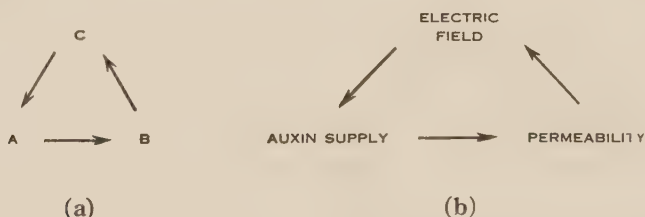


Fig. 11.—(a) General form of a simple feedback loop. (b) Possible feedback loop responsible for the electric oscillations described in this paper.

In addition to these, rhythms are sometimes found in systems living in an apparently unchanging environment as is the case for the oscillations described in this paper. Other examples are the autonomic to-and-fro or circular movements of elongating shoots, roots, tendrils, and rhizomes (Darwin 1880; Bennet-Clark and Ball 1951) and the periodic changes in colour and oxygen consumption of the fiddler crab in a controlled environment (Brown, Bennett, and Webb 1954). These endogenous rhythms have recently been reviewed by Bünning (1956). In the examples quoted above and in the case of the electric oscillations described in this paper the periods of the rhythms are of the order of minutes or hours.

Cases of overshoot and sometimes damped oscillation in biological systems following stimulation or a change in environment have also been described previously in the literature (Burton 1939; Idle 1955).

Little serious attempt appears to have been made to explain the mechanism of the internal processes which give rise to spontaneous rhythms which have periods of more than a few seconds. Bünning (1956) has described the rhythms as analogous to the movement of a pendulum. A comparison of this type is quite misleading. Consider a simple inertial system subject only to restoring forces (such as the

pendulum) which has a natural period of the order of minutes or hours. The amount of energy associated with this system would be so small that it would be dissipated in a small fraction of a period. For example, the energy of a 1-g body oscillating with an amplitude of 1 cm and period 5 min is only 2×10^{-4} ergs. In order to maintain a sinusoidal oscillation in such a system, energy losses would have to be replenished at exactly the correct rate.

The most likely source of slow sinusoidal oscillations is a negative feedback system. It is not proposed to consider here in any detail the behaviour of these systems as they have been studied fully in connection with servomechanisms and automatic control processes (Porter 1950). The following simple example (Fig. 11(a)) serves to illustrate the basic principles.

A , B , and C are three variable quantities which are interrelated in such a manner that a change in A causes a change in B which in turn causes a change in C . If now the change in C causes A to alter in such a manner as to oppose its original change, the system is said to be a closed-loop control system or a negative feedback system. In order to gain adequate control the signal fed back is usually amplified (by a factor known as the "feedback loop gain") and some external energy source is necessary. It will be seen that negative feedback tends to stabilize the system against disturbing influences and because of this is widely used in automatic control systems. A familiar example is the thermostat which is controlled by the temperature of the room, and which in turn governs the rate of flow of fuel to the furnace which heats the room.

Control by negative feedback can never be instantaneous as some time delay in the various processes in the feedback loop cannot be avoided. If either the time delays or the feedback loop gain is too large the system may become unstable and start to oscillate spontaneously. These oscillations are sinusoidal provided the quantities involved in the feedback loop are related linearly to one another. They may be of any period depending on the time delays occurring in the loop.

Thus it is seen that a system which under normal circumstances is kept stable and resistant to external disturbing factors by negative feedback may lose control and start to oscillate if one or more of the relationships in the feedback loop is altered. If the equilibrium of a system on the verge of instability is disturbed, it executes a damped oscillation before finally returning to the equilibrium state. The rather delicate balance which exists between stability and instability is familiar to those who handle feedback-controlled systems.

It will be noted that the root which generates electric oscillations has many properties in common with the systems discussed above. The electric field of the root may be almost steady for several hours when suddenly quite large oscillations which are approximately sinusoidal commence. If a plant producing a steady electric field is stimulated, damped electric oscillations often result.

It is therefore attractive to suggest that some automatically controlled process involving negative feedback is associated with the growing root. Under normal circumstances the system is stable but only just so, since transient changes are in the form of damped oscillations. Occasionally some change in the properties of the system causes it to become unstable thereby upsetting the control process.

Many self-regulating processes employing negative feedback are now recognized in the higher animals (Wiener 1948, Walter 1953). These include the homeostatic controls of such factors as temperature, balance, and blood pressure, as well as many others requiring conscious effort. So far very few similar controls have been found in the forms of life without nervous systems such as plants.

It is noteworthy that despite the frequent use of feedback processes in complex living systems, oscillatory behavior is seldom observed. Evidently the method of stabilizing these systems is effective over a considerable range of operating conditions.

In the present case not enough is known to say what aspects of the growth of the root are being controlled and what processes are involved in the feedback loop assuming that one is present. It seems unlikely that these processes are entirely electric. It is known that the electric impedance of cellular membranes has relatively large resistive and capacitive components ($0.25\text{ M}\Omega$ and $1\text{ }\mu\text{F}$ for 1 cm^2 in the case of *Nitella* (Blinks 1930, 1936)). These might conceivably be associated to form a long-period RC oscillator, say of the Wien bridge type, but it is difficult to see how this could be achieved. An amplifier of the correct gain would have to be available and the outputs of the individual cellular oscillators would have to be synchronized so as to give an observable oscillation in the external medium.

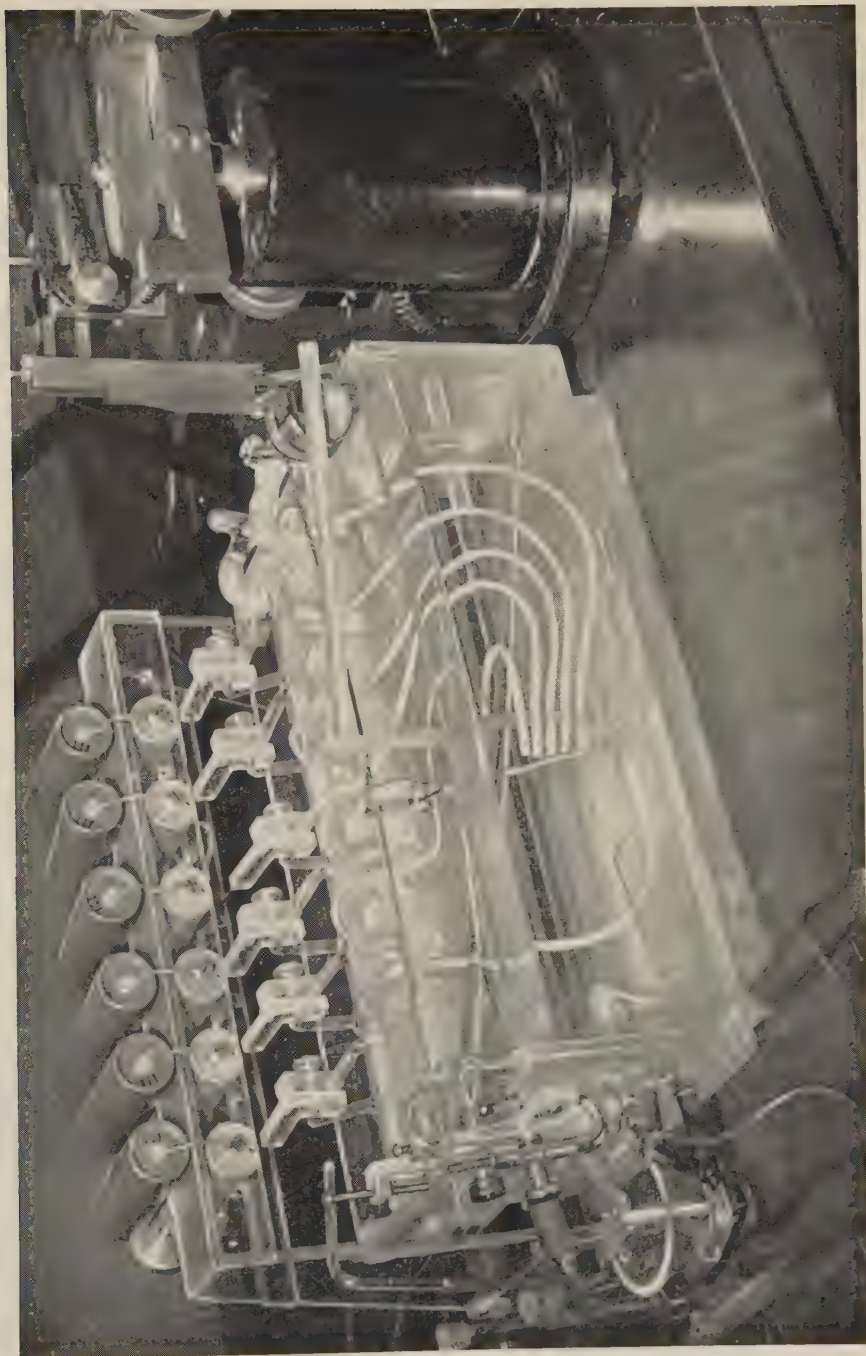
So far no other oscillatory variations in the bean root's behaviour have been found to be associated with the electric variations. It is of course possible that the electric field itself is not an active element in the loop but is merely coupled to some other process which is directly involved. If this is the case, the electric field is acting as a very convenient and sensitive indicator of the stability or otherwise of the control system.

To conclude this discussion some tentative suggestions as to the sequence of processes involved in the proposed feedback loop will be made. Suppose the auxin concentration in the elongating region of the root controls the permeability of the cell walls to salts (Thimann 1949). This would undoubtedly affect the electric resistance of the plant tissue and hence the magnitude of the electric currents flowing in the plant root and returning via a path in the external medium. If it is now supposed that the electric field modifies the rate of supply of auxin within individual cells or groups of cells to sites at which it affects permeability a feedback loop is thereby completed (Fig. 11(b)). Under some conditions the feedback system might become unstable and give rise to the observed oscillations. Sufficient feedback loop gain should be available because of the sensitivity of auxin action.

The possibility that the bioelectric field plays a part in the movement of auxin in plants was suggested many years ago (cf. Went 1932; Kögl 1933). Although the relationship is certainly not as simple as it first appeared, recent experiments by Schrank and Backus (1951) have suggested that the diffusible fraction of auxin may well be moved electrophoretically. The fact that swamping the water around the root with indoleacetic acid quenches the electric oscillation suggests that auxin plays some part in the feedback process.

Further search for other changes in the root while electric oscillations are being generated is necessary before the feedback system can be specified with any

ELECTRIC OSCILLATIONS GENERATED BY PLANT ROOTS



Photograph of measuring bath showing a bean root set up for measurement.

certainly. Some estimate of the time delays in the processes would then have to be made in order to see whether they are compatible with 5-min electric oscillations.

V. ACKNOWLEDGMENTS

The author wishes to express his thanks to Professor A. L. McAulay for his interest and helpful criticism of this work. Thanks are also due to Dr. D. B. Idle, University of Birmingham, who suggested the principle of the rate-of-growth meter. The assistance of Dr. D. Le Souef and Mr. D. Millwood, Department of Physics, University of Tasmania, in the design and construction of apparatus is also gratefully acknowledged.

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STUDIES ON PHYTOCIDES

II. TESTS OF CHLORINATED ARYLOXYMETHYLPHOSPHONOUS AND PHOSPHONIC ACIDS AS POISONS AND AUXINS

By C. G. GREENHAM*

[Manuscript received November 7, 1956]

Summary

Tests were made on pea epicotyls or oat coleoptiles with *o*-chloro-, *p*-chloro-, 2,4-dichloro-, and 2,4,5-trichlorophenoxyethylphosphonous acids. The first compound behaved like a weak auxin, the third like an inactive substance or anti-auxin (depending on the test), and the remainder like anti-auxins. The compounds usually showed some toxicity, which was far less than that of the corresponding carboxylic acids.

The monoethyl ester of *m*-chlorophenoxyethylphosphonic acid was inactive. Complete esterification of 2,4-dichlorophenoxyethylphosphonic acid resulted in greatly increased toxicity, whereas esterification of 2,4-dichlorophenoxyacetic acid did not affect toxicity at pH 5.

Possible reasons for the different physiological action of the phosphonous acids and carboxylic acids are briefly discussed.

I. INTRODUCTION

For the development of new poisons and auxins a standard procedure is to modify slightly the structure of a compound known to have suitable or nearly suitable properties. Thus 2,4-dichlorophenoxyacetic acid (2,4-D) has many suitable physiological properties, but is not always readily translocated in deep-rooted perennial weeds.

Partly in an effort to obtain a systemic poison, some phosphonic acid analogues of 2,4-D were tested. It was known that radiophosphate is well translocated, and it was hoped that a phosphonic compound, with some chemical analogies to phosphate, might be similarly well translocated. It was found (Greenham 1953 and unpublished data) that the phosphonic analogues tested, though possessing some toxicity, were not toxic enough for use as herbicides; it was, however, interesting to find that certain aryloxyphosphonic compounds possessed some auxin activity.

This paper reports tests of some chlorinated aryloxyethylphosphonous acids, and tests of some additional phosphonic acid derivatives. According to Kosolapoff (1950, p. 144) phosphonous acids are monobasic and are formulated as $RP(O)H(OH)$; therefore they should be closer than phosphonic acids $(RPO(OH)_2)$ in spatial structure to carboxylic acids, characterized in this paper by 2,4-D and some related carboxylic acids.

II. METHODS

For the pea epicotyl test, a modification of that of Galston and Hand (1949) was used. A pure line of Alaska peas was surface-sterilized with bleaching powder

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(10 g in 150 ml of water) for $\frac{1}{2}$ hr, washed, and soaked in water for 4 hr. The seed was then planted in damp vermiculite and incubated for 6–7 days at 25°C in the dark. Sections 6.6 mm long were cut at 8 mm distance from the top of the bend, all manipulations being done in dull red light. The sections were washed in distilled water before being incubated on a shaker in the dark at 25°C. Usually seven sections were used per dish containing 2 ml of solution, and there were two dishes per treatment.

For the coleoptile test, unhusked oat seed (var. Victory) was soaked for 2 hr in tap water, spread over damp vermiculite, just covered with more damp vermiculite, and germinated under dull red light for 3 days at 25°C. The dish was covered with a sheet of glass for the first 2 days. Sections 5.5 mm long were cut 2 mm from the tip, and were treated the same way as the epicotyl sections.

The phosphonous acids were initially dissolved in a little cold N NaHCO₃ solution (warm N NaOH may cause decomposition). The concentrations of the various compounds or auxins listed in Table 1 are in most instances equimolar with 2,4-D at 4.5×10^{-5} M (10 p.p.m.) or a multiple of that value. The solutions were buffered with M/100 potassium hydrogen phthalate or succinate (A.R. grade), and adjusted in pH with NaOH or HCl. For any one experiment all solutions were adjusted in pH to within 0.02 unit. Glass-distilled water was used throughout. Sucrose (2 per cent.) was included only in the first-done experiments (1 and 6), its main effect being to increase or to render uniform the elongations caused by strong auxins. For prolonged experiments the solutions were changed at least once daily, after the sections had been twice rinsed with the appropriate solution. Growth of microorganisms was never noticeable. Change in pH was less than +0.1 unit in 24 hr.

The method of measuring toxicity is a modification of one used previously (Greenham and Cole 1950). After the sections were measured for length, they were blotted dry, inserted in a jig, then impaled on two needle tips 3 mm apart (the primary leaves were removed from the oat coleoptile sections beforehand). Electrical resistance between the two needle tips was determined at 1 kc/s (R_{LF}) and at 1 Mc/s (R_{HF}); for the latter determination a Wayne-Kerr type B601 bridge followed by a selective amplifier and oscillograph, was used.

III. COMMENTS ON METHOD OF MEASURING TOXICITY AND ON LENGTH CHANGES

In this paper the ratio R_{LF}/R_{HF} is taken as the criterion of injury,* rather than the magnitude of R_{LF} (Greenham and Müller 1956). The ratio is taken because in many of the treatments length changes occur, affecting the number of cells between the fixed probe tips and therefore the measured value of R_{LF} (R_{LF} depends on the number of cell junctions between the needle tips and the extent to which the cells are injured). By using the above ratio, errors caused by variations in length are eliminated.

For sections in the control solutions, the length becomes constant within 24 hr. The R_{LF}/R_{HF} ratio increases slowly with time due to "salt" absorption (pro-

* It is proposed to publish a critical evaluation of this method in the near future.

vided there is no injury, e.g. from low pH), then decreases, particularly if sucrose and other metabolites are absent (Fig. 1).

For treated sections, cell elongations are complete within 24 hr; however, there may be subsequent shrinkage if there is loss of turgor (from extreme injury or death). The R_{LF}/R_{HF} ratios will behave quite differently from the control ratios if injury occurs. With toxic substances such as 2,4-D at $4.5 \times 10^{-5}M$ or higher concentrations, the ratio initially increases more rapidly than the control ratio, then

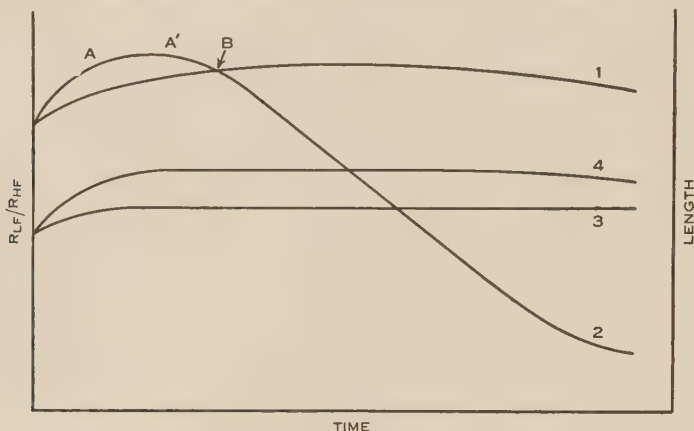


Fig. 1.—Curves illustrating changes in R_{LF}/R_{HF} ratio and in length over a period of 3 or 4 days (pea epicotyls, see text). The magnitude of the changes varies from experiment to experiment, hence the axes are not graduated. Curves 1 and 3: R_{LF}/R_{HF} ratio and lengths respectively for control sections. Curves 2 and 4: R_{LF}/R_{HF} ratio and lengths respectively for a slightly toxic auxin solution, e.g. 2,4-D at $4.5 \times 10^{-5}M$ (10 p.p.m.).

decreases and ultimately approaches the value 1 when the cells are dead.* The initial increase in the ratio over the control value is due to a temporary increase in R_{LF} (primary injury response—cf. Osterhout 1922, p. 46); the subsequent decrease in the ratio is due to a decrease in R_{LF} (secondary injury response) and to an increase in R_{HF} (leaching resulting from injury). With 2,4-D at $4.5 \times 10^{-3}M$ the ratio is usually 2 or less for pea epicotyls after 24 hr. In most of the present experiments 2,4-D at $4.5 \times 10^{-5}M$ has been included as a reference standard; this concentration is supra-optimal for cell elongations but usually suitable for causing injury. Not only section elongations but also the R_{LF}/R_{HF} ratios vary from experiment to experiment. Accordingly, when assessing toxicity in terms of this ratio, a comparison should be made with the value for control sections and with the value for sections treated with a standard concentration of a reference poison. With poisons weaker than 2,4-D, the rise and fall in the R_{LF}/R_{HF} ratio will be slower.

* Luyet (1932) showed that at death R_{LF} and R_{HF} were approximately equal. Hence the magnitude of R_{LF} in relation to the magnitude of R_{HF} gives a convenient measure of injury. Moreover, within the one population of sections, variations in R_{LF} are somewhat compensated for by appropriate variations in R_{HF} . The ratio R_{LF}/R_{HF} gives a reasonable criterion of injury, except possibly when sections have undergone maximal elongation due to treatment with a powerful auxin. Such maximal elongations are not encountered in the present paper. The ratio R_{LF}/R_{HF} has also given a suitable criterion for the assessment of frost injury (Greenham and Daday, unpublished data).

In the interpretation of the R_{LF}/R_{HF} ratios, an increase over the value for the control, e.g. point *A* or *A'* (Fig. 1) is generally to be regarded as an early toxic response (see below for an exception). If the value at any time is identical with that of the control, e.g. point *B*, there may or may not have been a temporary increase beforehand. However, if after a suitable period similar values for R_{LF}/R_{HF} are obtained for the control sections and for the sections treated at two widely differing concentrations of a poison, it may be assumed that the poison at least at the lower concentration has caused no injury (toxic response is mainly proportional to concentration if time is constant). If the values at any time are less than the control value, i.e. below point *B* (Fig. 1), injury has occurred.

In experiment 9, 2,4-dichlorophenoxymethylphosphonous acid at the highest concentration illustrates the exception referred to above, and gives the only such exception encountered in the investigation. Here the increase in R_{LF}/R_{HF} ratio is due to a decrease in R_{HF} , attributable to increased electrolytic concentration within the tissues; even the electrolytic conductance of the relevant solution was 2.05 times that of the control solution, due to the presence of the phosphonous acid. Increased electrolyte concentration within the tissue is regarded as evidence of "salt" uptake, so that in this case there is essentially no injury.

IV. RESULTS

From experiment 1, Table 1, it is seen that in a period of 24 hr the *o*-chlorophenoxymethylphosphonous acid was not toxic at 4.5×10^{-4} M and not obviously toxic at 4.5×10^{-3} M, whereas *o*-chlorophenoxyacetic acid was slightly toxic at 4.5×10^{-4} M (secondary injury response) and 2,4-D was slightly toxic at 4.5×10^{-5} M (primary injury response). In view of these results, some of the subsequent experiments for the determination of the toxicity of the phosphonous acids were done over longer time intervals.

With *p*-chlorophenoxymethylphosphonous acid (expt. 2, Table 1) there was slight toxicity at all concentrations within 94 hr, whereas 2,4-D at 4.5×10^{-5} M resulted in injury nearly to the point of death. From experiment 3 it is seen that the 2,4-dichlorophosphonous compound was somewhat toxic to pea epicotyls at 4.5×10^{-3} M but no more toxic than 2,4-D at 4.5×10^{-5} M. The 2,4,5-trichlorophosphonous acid was not obviously toxic within 21 hr (expt. 4), but was somewhat toxic at 4.5×10^{-3} M in 69 hr (expt. 5); 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) at 4.5×10^{-5} M and 2,4-D at the same molarity were considerably more toxic, and the former of these was more toxic than the latter. Usually 2,4-D is regarded as being more toxic than 2,4,5-T to herbaceous tissues, but the generalization does not hold in this test.

Experiment 6 shows that ethyl hydrogen *m*-chlorophenoxymethylphosphonate had no obvious toxic action (or even auxin action) at pH 5.0. The general high trend of the R_{LF}/R_{HF} ratios in this experiment is attributed to the use of a different batch of vermiculite which had been equilibrated with culture solution. Due to variability of the readings, the R_{LF}/R_{HF} ratio for the sections treated with 2,4-D at 4.5×10^{-5} M is just not significantly greater than that for the control sections.

TABLE I
BIOLOGICAL ACTIVITY OF PHOSPHONOUS AND PHOSPHONIC ACID ANALOGUES OF 2,4-DICHLORO-
PHENOXYACETIC ACID (2,4-D)
Pea epicotyl test, pH 5.0 with phthalate buffer, 25°C

Expt.	Duration (hr)	Compound	Concn. (M)	Length (mm)	R_{LF}/R_{HF}
1	24	Control		7.37	5.5
		<i>o</i> -Chlorophenoxyethylphosphonous acid	4.5×10^{-5}	7.39	5.3
		"	4.5×10^{-4}	7.45	5.5
		"	4.5×10^{-3}	7.95***	5.3
		<i>o</i> -Chlorophenoxyacetic acid	4.5×10^{-4}	8.25***†	5.1*
		2,4-D	4.5×10^{-5}	8.41***	6.0*
2	94	Control		7.86	5.76
		<i>p</i> -Chlorophenoxyethylphosphonous acid	4.5×10^{-5}	7.53***	7.53**
		"	4.5×10^{-4}	7.42***	6.98*
		"	4.5×10^{-3}	7.37***	5.81
		Diethyl 2,4-dichlorophenoxyethylphosphonate	6.3×10^{-4}	6.26***	1.03***
		2,4-D	4.5×10^{-5}	9.23***	1.40***
3	72	Control		7.24	6.8
		2,4-Dichlorophenoxyethylphosphonous acid	4.5×10^{-5}	7.26	6.2
		"	4.5×10^{-4}	7.18	6.8
		"	4.5×10^{-3}	7.21	4.6*
		2,4-D	1.8×10^{-4}	7.65***	1.3***†
		2,4-D	4.5×10^{-5}	8.06***	3.7**
4	21	Control		7.65	7.7
		2,4,5-Trichlorophenoxyethylphosphonous acid	4.5×10^{-5}	7.43*	7.8
		"	4.5×10^{-4}	7.46	7.7
		"	4.5×10^{-3}	7.30**	7.8
		2,4,5-Trichlorophenoxyacetic acid	4.5×10^{-5}	9.91***	9.6
5	69	Control		7.87§	6.3
		2,4,5-Trichlorophenoxyethylphosphonous acid	4.5×10^{-5}	7.64	5.9
		"	4.5×10^{-4}	7.64	5.3
		"	4.5×10^{-3}	7.42***	3.4***†
		2,4,5-Trichlorophenoxyacetic acid	4.5×10^{-5}	8.36***	1.3***†
		2,4-D	4.5×10^{-5}	8.45***	2.7***†
6	24	Control		7.57	8.5
		Ethyl hydrogen <i>m</i> -chlorophenoxyethylphosphonate	4.5×10^{-5}	7.55	9.4
		"	4.5×10^{-4}	7.54	9.4
		"	4.5×10^{-3}	7.34	9.3
		<i>m</i> -Chlorophenoxyacetic acid	4.5×10^{-4}	8.70***	8.1
		2,4-D	4.5×10^{-5}	9.93***	11.3

* Significantly different from control value at $P < 0.05$.

** Significantly different from control value at $P < 0.01$.

*** Significantly different from control value at $P < 0.001$.

† This value significantly different from preceding one at $P < 0.001$.

‡ This value significantly different from preceding one at $P < 0.01$.

§ For lengths, a difference of 0.24 is significant at $P < 0.05$.

Experiment 2 also shows that the diethyl ester of 2,4-dichlorophenoxyethyl-phosphonic acid* is highly toxic, whereas the ethyl half-ester showed comparatively little toxicity (Greenham 1953, and unpublished data). In fact, experiment 2 probably does not do justice to the toxicity of the di-ester, because the sections appeared dead within 24 hr (dead epicotyl sections appear characteristically dull white). The apparent shrinkage of the sections is attributed to rapid death followed by loss of turgor (Stiles 1930).

TABLE 2

BIOLOGICAL ACTIVITY OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) AND ITS ETHYL ESTER
Pea epicotyl test, pH 5.0 with phthalate, 25°C

Expt.	Duration (hr)	Compound	Concn. (M)	Length (mm)	R_{LF}/R_{HF}
7	22	Control		7.73	4.2
		2,4-D	4.5×10^{-5}	8.71***	5.4**
		2,4-D ethyl ester	4.5×10^{-5}	8.50***	5.0
		2,4-D	3.6×10^{-4}	7.47	1.3***
		2,4-D ethyl ester	3.6×10^{-4}	6.73***†	1.2***
	66	Control		7.74	7.0
		2,4-D	4.5×10^{-5}	8.53***	7.0
		2,4-D ethyl ester	4.5×10^{-5}	8.31**	7.4
		2,4-D	3.6×10^{-4}	7.31*	1.3***
		2,4-D ethyl ester	3.6×10^{-4}	6.64***†	1.3***

* Significantly different from control value at $P < 0.05$.

** Significantly different from control value at $P < 0.01$.

*** Significantly different from control value at $P < 0.001$.

† This value significantly different from preceding one at $P < 0.001$.

‡ This value significantly different from preceding one at $P < 0.01$.

Esterification of 2,4-D, however, did not significantly affect its toxicity at pH 5 (Table 2), although the esterification did depress elongation at the higher concentration. In this experiment the epicotyl sections were more resistant than usual to 2,4-D at 4.5×10^{-5} M, due probably to the use of a different batch of seed.

The point is now made that growth inhibition may be caused either by toxic action or by anti-auxin action. Therefore, as none of the phosphonous compounds tested were particularly toxic, it is concluded that those compounds which were responsible for reduced section elongation in comparison with the controls behaved like anti-auxins (competition with endogenous auxin).

Accordingly, as regards "auxin" activity of the phosphonous compounds, the *p*-chloro- and the 2,4,5-trichloro- compounds acted like anti-auxins, the 2,4-dichloro- compound was inactive to pea epicotyls, and the *o*-chloro- compound acted like a weak auxin (expts. 1-5). "Auxin" activity within these compounds is in no way comparable with that in the corresponding carboxylic acids.

* The term "methyl" is used here in place of the former term "methane", to comply with recommended changes in nomenclature (Editorial Report on Nomenclature, 1952, *J. Chem. Soc.* 1952: 5123).

It was thought that lack of activity on the part of the 2, 4-dichloro- phosphonous compound might possibly be due to testing under unfavourable conditions; accordingly a test was done under more acid conditions and in the presence of succinate buffer. However, no response was obtained with the pea epicotyls (Table 3, expt. 8), whereas the oat coleoptiles responded as to an anti-auxin (expt. 9). It is not unusual for a substance to act differently with different test species. The low R_{LF}/R_{HF} ratios obtained throughout experiment 8 suggest general toxicity as a result of low pH

TABLE 3
BIOLOGICAL ACTIVITY OF 2, 4-DICHLOROPHOXYMETHYLPHOSPHONOUS ACID
pH 4.0 with succinate buffer, 25°C

Expt.	Conditions	Duration (hr)	Compound	Concn. (M)	Length (mm)	R_{LF}/R_{HF}
8	Pea epicotyl test	22	Control		7.08	3.7
			2,4-Dichlorophenoxyethylphosphonous acid	4.5×10^{-5}	7.21	3.3
			"	4.5×10^{-4}	7.08	3.6
			"	4.5×10^{-3}	7.02	3.3
			2,4-Dichlorophenoxyacetic acid	4.5×10^{-5}	7.79***	3.1
9	Oat coleoptile test	26	Control		8.01	3.2
			2,4-Dichlorophenoxyethylphosphonous acid	4.5×10^{-5}	7.89	—
			"	4.5×10^{-4}	7.84	3.4
			"	4.5×10^{-3}	7.26***	3.7*
			2,4-Dichlorophenoxyacetic acid	4.5×10^{-5}	8.50***	3.7*
10	Oat coleoptile test	22	Control		6.99	—
			2,4-Dichlorophenoxyethylphosphonous acid	4.1×10^{-3}	6.83*	—
			2,4-Dichlorophenoxyethylphosphonous acid + 2,4-Dichlorophenoxyacetic acid	4.1×10^{-3}	7.28****†	—
				1.13×10^{-5}		
			2,4-Dichlorophenoxyacetic acid	1.13×10^{-5}	7.79****†	—

* Significantly different from control value at $P < 0.05$.

** Significantly different from control value at $P < 0.01$.

*** Significantly different from control value at $P < 0.001$.

† This value significantly different from preceding one at $P < 0.001$.

(similar evidence of toxicity is obtained with phthalate at pH 4). It is not feasible to compare the R_{LF}/R_{HF} ratios in experiments 8 and 9, as different tissues are involved. It has already been noted that the increase in the R_{LF}/R_{HF} ratio for the 2,4-dichlorophosphonous acid at the highest concentration in experiment 9 is not attributable to injury.

The classical method of studying anti-auxin behaviour is based on the inhibition of growth caused in the presence of a known auxin (McRae and Bonner 1953). The quantities of phosphonous acids available permitted only the additional

experiment 10. In this experiment, $(a-b) > (c-d)$ at $P < 0.001$, where a is the length of the sections treated with 2,4-D, b is the length of the control sections, c is the length of sections treated with 2,4-D plus the phosphonous acid, and d is the length of the sections treated with the phosphonous acid. This significant difference, while not being conclusive proof, supports the supposition that the 2,4-dichloro- phosphonous acid is an anti-auxin.

V. DISCUSSION AND CONCLUSIONS

It is seen that replacement of the carboxyl radical with the phosphonous radical changes substances with generally high toxic and auxin activity into substances with low activity (frequently substances with low auxin activity behave as anti-auxins, *vide* Leopold 1955, p. 168). It was previously shown that replacement of the carboxyl radical with the phosphonic radical (Greenham 1953; Veldstra *et al.* 1954) or the sulphate or sulphonic radicals (Veldstra *et al.* 1954) resulted in lessened activity. The present results with the half-ester of *m*-chlorophenoxy-methylphosphonic acid also supports this claim.

Since the above measurements were done, dissociation constants (determined by spectral titration) have become available for some of the compounds tested above. They are 2,4-D 2.8, *p*-chlorophenoxy-methylphosphonous acid 0.8, 2,4-dichloro- analogue 0.8, 2,4,5-trichloro- analogue 0.7, and ethyl hydrogen 2,4-dichlorophenoxy-methylphosphonate 0.7. The half-ester of *m*-chlorophenoxy-methylphosphonic acid probably has a similar value.

The pK values are identical for the *p*-chloro- and the 2,4-dichloro- phosphonous compounds, so that within the series of phosphonous compounds tested, physiological activity is not correlated with pK value. Within the series, positioning of the chlorine atom on the benzene ring affects physiological activity.

The low dissociation constants for the phosphonous and phosphonic acids tested means that they are almost completely dissociated at physiological pH, as was claimed by Veldstra *et al.* for the sulphonous analogues mentioned above. This raises the question as to whether the reduced activity of the phosphonous and phosphonic acids, in comparison with allied carboxylic acids, depends on their greater dissociation or on a lower biochemical reactivity of their acid radicals. An allied problem is whether 2,4-D acts in the ionic or molecular state.

Certainly Veldstra and Booi (1949) and Veldstra *et al.* (1954) consider that 2,4-D acts in the ionic state, as do Brian and Rideal (1952) for 2-methyl-4-chlorophenoxyacetic acid. However, it is felt that such claims sometimes overlook the increased activity of 2,4-D caused by lowering the pH of the external medium, although Brian and Rideal rightly point out that external pH may influence the behaviour of plasma membranes and of protoplasmic constituents. Nevertheless, the results of this paper show that suppression of the ionization of the dichloro- phosphonate compound by full esterification greatly increased its toxicity.

Nevertheless, degree of dissociation is not the only factor influencing toxicity. Another important factor influencing biological activity is the hydrophilic/lipophilic (H/L) ratio (Veldstra 1949). Veldstra and Booi (1949) claim that 2,4-D has a

suitable H/L ratio for auxin activity, and possibly for that reason the auxin activity of 2,4-D is not increased by esterification. Since structural modification by esterification eliminates ionization but simultaneously alters the H/L ratio, it is possible that maximum biological activity depends on a compromise between pK value and H/L ratio.

The questions raised in the preceding paragraphs cannot be solved within the scope of the present paper, and will be investigated subsequently.

VI. ACKNOWLEDGMENTS

The above phosphonous and phosphonic compounds were synthesized by Dr. M. H. Maguire and Dr. G. Shaw (1957), University of Technology, Sydney, as part of a collaborative programme. The ethyl ester of 2,4-D was supplied by Timbrol Ltd., Rhodes, N.S.W.

The dissociation constants were determined by Dr. J. N. Phillips, Division of Plant Industry, C.S.I.R.O., Canberra.

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WOUND HEALING OF THE POTATO TUBER IN RELATION TO INFECTION BY *PHYTOPHTHORA INFESTANS* (MONT.) DE BARY

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[Manuscript received December 24, 1956]

Summary

The protective action of healing potato tuber tissue against *Phytophthora infestans* (Mont.) De Bary is made manifest in two ways:

(i) In the tissues adjacent to the injured surface the speed of reaction to the pathogen, as evidenced in production of necrotic changes, is altered; it increases with increasing time interval between injury and infection. In close association with this, development of aerial mycelium on the inoculated face decreases, sporangiophore production ceasing completely after an interval of 4 days.

(ii) A marked decrease in susceptibility to infection occurs in the healing face; this becomes obvious before any cell divisions take place in the tissues which will form wound periderm. A statistical analysis showed that the probability of an individual spore being able to penetrate the protective barrier of wound periderm is decreased by at least 92 per cent. when an interval of 6 days has occurred between injury and exposure to the parasite, the tubers being kept at temperatures around 20°C.

The results are discussed in the light of the following hypotheses: (1) as a result of mechanical injury there is an increase in sensitivity towards the parasite produced in the tissues adjacent to the injured face (change of reactivity towards suprasensitivity), and (2) the development of a mechanical factor (possibly deposition of suberin in the cell layer adjacent to the injured surface) makes it more difficult for the parasite to gain a footing in the host tissue (increase of "passive resistance" to infection).

I. INTRODUCTION

Peridermal tissues are commonly regarded as effective barriers against infections by pathogenic microorganisms. Infections of potato tubers by *Phytophthora infestans* (Mont.) De Bary, for example, are considered to occur only through bud initials, lenticels, stolons, or wounds, the unbroken skin being impenetrable to fungal or bacterial parasites. This was confirmed by previous inoculation experiments carried out with tuber initials. As soon as the epidermal cells of the young tuber had undergone two divisions in peridermal formation, infection through the skin no longer took place, (Müller, unpublished data).

A similar effect is produced by peridermal tissues which are developed in response to mechanical wounding, e.g. in potatoes. Where a continuous layer of wound cork has been established, adjacent tissues have been found to be largely protected against subsequent infections whether bacterial or fungal. The majority of writers on this subject attribute this protection to the deposition of suberin in the wound periderm cells. It follows then that the more quickly the wound cork is developed, the greater is the protective effect; but the influence of environmental

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factors on the speed of wound periderm formation causes a considerable variation in the time necessary for the development of this protective mechanism (Appel 1906; McKee 1954, 1955; Smith and Smart 1955).

The protective effect of wound periderm against *Phytophthora* infection has not previously been investigated systematically, though Meyer (1939) mentions that 4 days after wounding, the parasite is still able to penetrate into the parenchyma of the tuber, but the reaction of the tuber to the parasite had changed. Necrotic changes in response to infection appeared much sooner in a tuber which had been cut 3 days before inoculation, than in one inoculated immediately after cutting; also fructification was much less in the former than in the latter. In the course of wound periderm formation an accumulation of phenolic substances occurs in the cells adjacent to the cut face, and Meyer concludes that the inhibition of the parasite is due to these phenolic or tannin-like substances.

McKee (1955) attributes the inhibition of *Fusarium caeruleum* (Lib.) Sacc. and *F. avenaceum* (Fr.) Sacc. in sap from wounded tissue to an increased concentration of solanine occurring in wounded tissue after 6 days.

Numerous infections of potatoes with *Phytophthora* occur around harvest time, when soil is wet and contains a high proportion of infective spores and where wounds have been inflicted during lifting. It therefore seemed worthwhile to investigate quantitatively the relationship between wound periderm formation, the probability of infection, and the clinical reaction produced by the tuber in response to those hyphae which do succeed in penetrating into the tissue.

II. MATERIALS AND METHODS

Sebago variety of tubers was used throughout these experiments; this variety is susceptible to all known *Phytophthora* strains. The *Phytophthora* strain used was of B group and was very virulent on Sebago tubers (belated necrosis and abundant fructification seen on inoculated faces).

Dishes lined with moist blotting-paper and covered with a glass sheet were used as containers. The tubers were cut longitudinally on different days in order to have a variety of time intervals between cutting and inoculation. These were placed in the containers, with the cut surface upwards, kept at 20°C, and, finally, all were inoculated at the same time with the one inoculum preparation.

The following tests and observations were made:

- (i) The suberization of the cut surface, and the appearance of cell divisions in the adjacent layers.
- (ii) The probability of individual spores being able to penetrate through the changing surface layer.
- (iii) The reaction of tuber parenchyma to the invading hyphae of the parasite.

Methods used were as follows:

In (i) the tissues adjacent to the healing face were hand-sectioned, and the sections examined microscopically for suberization and cell division in surface layers. The conventional stains were used to assist observation.

In (ii) the tubers were left for varying times after cutting, then slices 1 mm thick were taken from the cut surface of each. From these slices disks 10 mm in diameter were cut with a cork borer, and placed at random on wet filter paper in dishes, with the healing surface upward. Each dish contained 56 disks, random samples taken from the slices which had been cut from healing surfaces after intervals of 1, 3, 6, and 10 days. Zoospore suspensions of different densities were sprayed on these disks, the dishes being rotated during spraying to ensure equal distribution of the spores. After 24 hr the disks were inverted to allow for easier exit of fructifications. After a further 4 days the diseased and non-diseased slices were counted and the results obtained analysed statistically.

In (iii) filter-paper disks, 5 mm in diameter, were soaked in a dense zoospore suspension and placed on the cut surfaces, five to each half tuber. The effect was recorded in terms of necrotic changes on the inoculated face and the aerial mycelium produced thereon by the parasite.

III. RESULTS

Microscopical examination of the cut surfaces showed that suberin formation had started earlier than 24 hr after cutting the tubers. The first cell divisions, however, were observed much later, the earliest appearing after 2 days. During the following 2-3 days, the number of cell divisions increased sharply so that a nearly continuous layer of divided cells had been formed by the end of this time. These observations are in good conformity with those of other workers (Appel 1906; Artschwager 1927; Smith and Smart 1955).

The response to infection of the tissues adjacent to the cut surface change markedly with increasing time after cutting. This can be seen in Table 1. The tubers inoculated immediately after cutting showed no definite necrotic changes at points of inoculation; the majority of cells, in this inoculated area, remained turgid for 6 days, and abundant fructification had occurred. The tubers, which were inoculated after 1 day's interval between cutting and inoculating, showed a marked browning on the inoculated face and the fructification was much less than in the case of the freshly cut tuber examined 6 days after inoculation.

It can also be seen from Table 1 that the greater the interval between cutting and inoculating, the more striking was the difference in the response of the tissues to the parasite. Accordingly, in the tubers which had had an interval of 5 days between cutting and inoculating, only some necrotic spots were seen at the point of inoculation and no fructification was produced. These results are consistent with those obtained by Meyer (1939).

Within the tubers also, certain differences were observed between those inoculated immediately after cutting and those which had been subjected to an interval between cutting and inoculation. In the former no necrotic changes were seen in the tissue; where an interval had occurred, however, the tissues showed browning until in the case where 5 days had elapsed before inoculation, brown traces with sharply bordered edges were seen in tissue near the inoculated face. Thus there was reason to assume that even the tissue which lies at a distance from these healing

layers is also to some extent, and in some way, affected by those changes taking place at the cut surface.

Accordingly a further test was made by quartering some of the non-inoculated half tubers already used, and uniformly inoculating those freshly cut surfaces which lie at right angles to the original healing surfaces. No marked effect was seen on the freshly cut surface, except for a slight reduction of fructification in the layers

TABLE 1

INTERACTION BETWEEN HOST TISSUE AND PATHOGEN AFTER INCREASING INTERVALS BETWEEN CUTTING AND INOCULATION OF THE TUBERS

Time after Cutting (days)	Necrotic Changes of Host Tissue after 6 Days		Fructification of the Fungus after 6 Days
	At Cut Surface	Below Cut Surface	
0	None	None	Abundant
1	Diffuse brown discoloration in and around inoculated areas	Diffuse brown discoloration	Scattered; aerial mycelium rather poor
2	Necrotic patches more or less sharply defined	Not examined	Similar, but markedly less than before
3	Necrotic patches to necrotic spots	Not examined	Very little to none
4	Necrotic spots only	Diffuse necrotic traces near the inoculated face	None
5	Fewer necrotic spots	Sharply defined necrotic traces	None

closely adjacent to the wound periderm tissue. This negative result suggests two possibilities, either (1) that the pathogen, in passing through the region of developing wound periderm, undergoes some change which influences the interaction between host and pathogen, so that necrosis occurs much earlier in the adjacent tissue; or (2) that after a fresh surface is cut, the conditions within the tissues concerned are altered and the original equilibrium between host and pathogen is restored. At present no decision between these alternatives is possible.

Tubers which had been inoculated 5 days after cutting showed only a few necrotic changes in the inoculated area. This indicated that only a very small proportion of the spores placed on the cut surface had been able to penetrate into the tuber parenchyma, and the majority of these had not established an infection. We may conclude, therefore, that with increasing time after cutting the probability

(P_i) of an individual spore being able to penetrate the healing surface decreases considerably. This was proved by experiments carried out as follows:

Three separate experiments were set up, using five or six dishes in each case, with disks from the differently "treated" tubers distributed at random among the dishes. These were divided into three lots and inoculated with "light", "medium", and "heavy" doses of the pathogen. As Table 2 shows, the number of successful spores decreases markedly with the time which elapses after cutting and before inoculation. After 10 days, although a heavy spore load was applied, only a small proportion were successful in penetrating.

TABLE 2

PERCENTAGE OF INFECTION ON TUBER DISKS EXCISED FROM THE CUT SURFACES AT DIFFERENT INTERVALS AFTER CUTTING

Spore Load	Days after Cutting				
	0	1	3	6	10
Light	90.5	84.0	38.9		
Medium	100.0	100.0	50.8	16.1	
Heavy			60.4	32.4	13.5

In order to have a quantitative measure of the probability of a single spore becoming infective, a statistical analysis of the data was made. It showed variation between the replicates in excess of the errors of chance. Nevertheless, the trend to progressively lower infection with increase in period after cutting is quite significant with all the intensities of spraying. An estimate of the relative likelihood of infection is given by $\log_e p$, where p is the proportion of tuber slices which did not show infection. Through the three statistical estimates of the experimental values, the three intensities of spraying at 3 days and the two estimates at 6 days, combined estimates over all dosages were determined for the relative probabilities of a spore causing infection. The value at 3 days was arbitrarily taken as 1.00. Relative values are tabulated as follows:

Days after cutting and before inoculation	0	1	3	6	10
Relative probability of spore causing infection	4.76	3.71	1.00	0.36	0.14

From these results it is seen that inoculation at zero interval after cutting is *c.* five times as effective as inoculation 3 days after cutting, and more than 30 times as effective as inoculation 10 days after cutting.

The actual differences must necessarily be greater than the above for the following reasons: in preparing and spraying the disks, freshly cut surfaces are exposed to the pathogen and these also would become infected, obviously affecting the final values given above. The 0.14 value at 10 days could be only the result of

these freshly cut surfaces and if this is subtracted from each value, then the relative differences of probability become much greater than shown in the above tabulation.

In the graph shown in Figure 1 these values and observations on wound periderm formation and the interaction between host and parasite are plotted against each other. From this it can be seen that a marked reduction of P_i occurs before the first cell divisions begin; the same is true for the intensity of fructification of the pathogen on the cut surface. On the other hand, there is a marked increase in the rapidity of appearance of the first necrotic changes in response to an infection. This indicates that the cell division itself in wound tissue is not the only, and perhaps not even the essential, factor in the protective mechanisms produced after mechanical injury. The suberization of these outer cells, however, coincides with the first changes inhibitory to the pathogen.

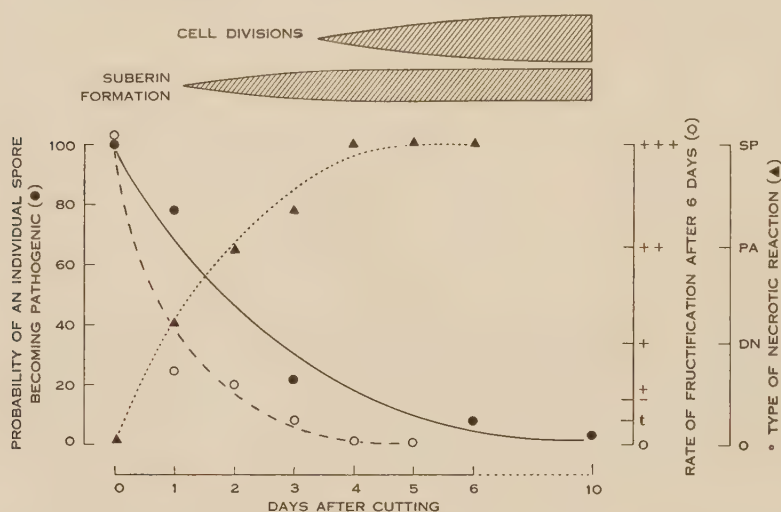


Fig. 1.—Diagram illustrating the time relationship of wound periderm formation, reaction of the wounded tissue to infection, fructification of the pathogen, and the probability of an individual spore becoming pathogenic. Onset and intensity of cell division and suberization are indicated by the hatched diagrams above the graph. 0, no necrotic change; *DN*, diffusing necrosis; *PA*, necrotic patches; *SP*, necrotic spots (clearly defined); + + +, abundant fructification; *t*, traces of fructification; —, no fructification—other symbols on this axis are intermediates between the extremes.

IV. DISCUSSION

It has been found that there is a gradual increase in protection against pathogenic infection in the tuber tissues which are changing to form wound periderm. This protective effect increases with time, the first marked changes being seen after 24 hr. This result is in good accord with those obtained by previous authors working with bacterial diseases.

The most interesting outcome of this work is that the protective action of healing tissue is made manifest in two ways: firstly, in a change in the reaction of the host tissues, adjacent to the wounded surface, towards infection by the invading

pathogen, and secondly in that the proportion of spores able to establish an infection decreases as the time interval between injury and inoculation increases.

Meyer (1939) suggested that the decrease of the activity in the growth of the pathogen was due to an accumulation of phenolic substances in those cells which are changing into wound periderm. McKee (1955), so far as *Fusarium* infections are concerned, suggests that the presence of solanine, which he found to be present in greatly increased amounts in tissues below the cut surface, is the cause of the inhibition of the pathogen. But there is a striking correlation between the rate of necrotic change of the infected tissue and the decrease in growth activity of the pathogen. This means that the greater the necrotic disintegration of the host cell, in response to the infection, the greater is the inhibiting effect on the pathogen. This would indicate that within the tissue affected by the mechanical injury, an increase of sensitivity and with this an increase of reactive resistance towards the pathogen takes place. The simplest explanation of this sensitizing effect may be the assumption that in the actual healing process a toxic principle is produced which is the precursor of, or even identical with, that factor which retards the pathogen, and which at the same time causes the disintegration of the host cell. Whether this hypothesis is in conformity with the truth must be left for further research to decide.

As far as the reduction of the number of spores able to establish an infection is concerned, there is good reason to assume that here the protective effect is a completely passive one (mechanical or chemical or both). In the early stages of healing there will be gaps between the groups of dividing cells forming wound periderm; with the passage of time these gaps become fewer and smaller until finally the periderm tissue is continuous. Therefore, the chance of penetration by the pathogen automatically becomes less as the time between injury and contamination increases. Here then the whole question would be reduced to a statistical problem, namely the combined effect of both the number of infective units, each of which is a possible initiator of disease, and the total area of sites available for infection, on the clinical result of contamination of a susceptible host with a pathogen. The question, however, whether the actual protective mechanism is solely a mechanical one (suberization of the outer walls of wound periderm?) or a chemical one which locally prevents, by virtue of its toxicity, penetration of the parasite into the host tissue, is again a problem which must be left for further research to decide.

The same principle must be taken into account when we consider the probability of an infection being established under field conditions during harvest time. It is self-evident that both the amount of viable individuals of the pathogen in the soil and the speed with which wound periderm formation proceeds, co-determine the incidence of tuber blight due to wound infections which take place after the tubers have been lifted. The greater the latter the greater is the quantity of spores necessary to ensure that an infection becomes established. But no direct proportionality between these two factors can be expected. According to our results, the relative importance of the number of spores per unit volume of soils depends largely on the speed with which wound periderm is formed. In the late autumn, wound periderm formation proceeds rather slowly and therefore at that time relatively great differences of spore load are somewhat insignificant unless the density of the pathogen in the soil is

comparatively low. On the other hand, in the early autumn when temperatures are higher and wound periderm develops relatively quickly differences of spore load even at high levels may have a great influence on the incidence of tuber blight due to wound infection. Under these circumstances reduction of the pathogen's population can have a marked effect on the incidence of blight infections established after the tubers have been lifted. This may be one of the reasons why the preventative effect of premature killing of foliage and stalks by spraying the crop with toxic chemicals, for instance sulphuric acid, in order to stop spore production, varies so much with date of spraying.

V. ACKNOWLEDGMENTS

The author wishes to acknowledge with thanks the assistance received from Mr. G. McIntyre with statistical treatment of data, Miss J. Friend with technical help, Mrs. S. M. R. Jerome in drafting the manuscript, and Mr. G. C. Greenham in reading it.

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THE DEVELOPMENT AND REPLACEMENT OF PELAGE HAIRS IN THE
BANDICOOT *PERAMELES NASUTA* GEOFFROY
(MARSUPIALIA: PERAMELIDAE)

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[Manuscript received November 20, 1956]

Summary

A histological study has been made of the development and replacement of pelage hairs in the bandicoot, *Perameles nasuta* Geoffroy. Skin samples were taken from the mid-lateral region of the trunks of 18 specimens from eight different litters at estimated ages ranging from birth to 526 days. Six specimens were sampled more than once.

At birth pelage hair follicles are absent, and the first follicles appear in the mid-lateral region about 11 days after birth. The first hairs emerge in this region at about the 40th day, and at 48 days all, or most, of the follicles of the first hair cycle have emerging hairs.

The follicles which develop during this first hair cycle are arranged in groups. Within each group the following follicles may be distinguished: a single original central primary follicle, a pair of original lateral primary follicles, and a number of original secondary follicles. All these original follicle types arise directly from the epidermis and open independently on the skin surface; they are to be distinguished from the derived follicles arising late in the first cycle and in succeeding cycles. Derived follicles develop by branching from existing follicles and share a common orifice with their parent follicle.

The special features in the development of the different types of hair follicles of the first hair cycle are described, and their rates of development are compared.

Before the end of the first hair cycle the derived follicles begin to arise by branching from all follicles, except the central primary of each group, a method of follicle replacement which apparently has not been previously described in mammals.

As successive hair cycles occur, further follicles arise by branching from earlier-formed lateral primary and secondary follicles, and from a few original central primary follicles. This results in the formation of bundles of closely associated follicles with a common orifice, and an increase in size of the follicle group. New original central primary hairs, which merely replace older hairs of similar type, develop in follicles arising from the bases of resting original central primary follicles; they emerge on the ectal, or grooved, sides of the old hairs. The hairs which develop in the derived central primary follicles are circular in cross section and appear to be similar in type to those of secondary follicles.

I. INTRODUCTION

The bandicoot, *Perameles nasuta* Geoffroy, is a typical marsupial in that the young are born at a rather early stage of development and are reared within a pouch. Because of this the pouch young have proved to be very favourable material for studying the early stages in the development of the hair coat; in eutherian mammals this development occurs *in utero*. *P. nasuta*, on the other hand, is born before the

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pelage hair follicles are formed. Periodic surgical excision of skin samples has made it possible to follow the entire development, and the later history; of the hair follicles in one and the same specimen.

Since the structure and development of the skin and hair in marsupials appears to be fundamentally similar to that of eutherian mammals, the use of the more favourable marsupial material for the study of many of the basic problems associated with the biology of the mammalian integument seems to be fully justified.

The only known study of marsupial hair development seems to be that of Gibbs (1938) on the development of the skin and hair in pouch young of the brush-tailed possum *Trichosurus vulpecula* Kerr. *T. vulpecula* and several other marsupials are exceptional in that practically the whole of the skin is covered by a coat of hairs before the vibrissae emerge (Lyne, unpublished data). Gibbs drew attention to this unique character of hair formation in *T. vulpecula* and suggested that it is probably associated with the development in the pouch. However, Gibbs was not aware that only very few marsupials show this early development of the first pelage hairs; the shedding and replacement of these hairs, which take place at an early age (Lyne, unpublished data), appear to have been completely overlooked.

The grouping of hair follicles in the skin of marsupials has received little special attention in comparative studies of the mammalian coat. Limited studies have been made by de Meijere (1894) on a number of different marsupials, by Sweet (1907) on *Notoryctes typhlops* Stirling, by Gibbs (1938) and Bolliger and Hardy (1944) on *T. vulpecula*, and by Hardy (1947) on 13 Australian marsupials. In Hardy's study no detailed consideration was given to ontogenetic or phylogenetic relationships, or to the relation between the follicle types in the skin and the hair types in the coat. Hardy pointed out, however, that her study of the adult hair follicle group-arrangement made abundantly clear the need for examining the development of follicle groups in the pouch young of marsupials in order to interpret the adult structure.

The presence of two distinct hair types—large grooved hairs and small circular hairs—in the bandicoots *Isodon obesulus* Shaw & Nodder and *Perameles gunnii* Gray, described by Lyne and McMahon (1951), suggested that either of these marsupials, or related species, would be ideal for the study of the relation between hair types and follicle types.

Although de Meijere (1894) briefly reported the branching of hair follicles in monotremes, marsupials, and various eutherian mammals, the origin of bundles of hair follicles appears to have been overlooked by all but a few authors. Tänzer (1926) described the branching of wool follicles in Karakul sheep and Hardy and Lyne (1956b, 1956c) have shown that the bundles of wool follicles in Merino sheep are formed by the branching of follicles which arise directly from the epidermis. The significance of these findings suggested the advisability of further investigation of some of the problems of hair follicle branching.

In many eutherian mammals the pelage hair follicles exhibit cyclic activity in alternating periods of growth and rest. This aspect of hair growth in *P. nasuta*, not previously described in any marsupial, is briefly outlined in this paper. The particular method of follicle replacement, associated with the hair cycles, has not been previously described in mammals.

II. MATERIAL AND METHODS

(a) *Animals*

During the collecting period (1954–55), 45 adult or nearly adult *P. nasuta* were trapped near Sydney, N.S.W., and were maintained in captivity in this Laboratory. Of these, 20 were females and nine were carrying young in the pouch. Although several litters have been successfully reared to maturity, *P. nasuta* has not, to date, been bred in captivity. Specimens of one litter (No. 2) reared in captivity and used in the present investigation are shown in Plate 1. These specimens had their eyes open at 48 days and were first seen out of the pouch 3 days later. The specimens of another litter (No. 6) had their eyes open at about 45 days after birth and were first seen out of the pouch at 55 days.

A total of 26 pouch young was available for the present investigation and of these 11 were females and 11 were males while four were of unknown sex. In addition to the pouch young, two advanced intra-uterine young were obtained from an adult which died after 11 days in captivity.

The pouch young were measured, sexed when possible, and live weights were obtained at intervals after the period of attachment to the teat. Length of the tail, ear, pes, and manus were recorded in a manner similar to that described by Lyne (1951) and two additional measurements, the head length and crown-rump length, were also obtained.

(b) *Estimation of Age*

The age of eight specimens was estimated from the measurements of two newly born young, given by Hill (1897), and repeated observations on four of these were used to determine the regression of age on the six measurements of length described above. A cubic regression line was fitted for each measurement, and, from the six estimates of age so obtained, a mean was obtained by weighting each estimate with the inverse of its error variance. The accuracy of these estimates was satisfactory for ages of 50 days or over. Within the range 0–50 days a cubic regression line for age on head length was used.

(c) *Skin Sampling and Histological Methods*

A total of 53 post-natal skin samples, taken from the mid-lateral region of the trunk (referred to as the midside region), was obtained by biopsy or immediately after death from 18 specimens from eight different litters. Twelve specimens were sampled once at estimated ages ranging from 1 to 40 days, and six specimens were sampled more than once at estimated ages ranging from 4 to 526 days. Full details of the specimens sampled are given in Table 1.

The midside skin samples were taken from either side of the trunk. When a specimen was sampled more than once the right and left sides were sampled alternately. The third and later samples were taken from a position either slightly anterior or posterior to the midside position. To avoid sampling the same position a second time the scar resulting from the previous sampling was tattooed before it disappeared. All the midside skin samples were fixed in Zenker's fluid. One entire

pouch young was fixed in 5 per cent. formalin; the other pouch young and the intra-uterine young were fixed in Bouin's fluid. Most of the skin samples were divided into two or more pieces after clearing in cedar-wood oil. Usually two pieces

TABLE 1
DETAILS OF SPECIMENS SAMPLED*

Litter No. and Estimated Date of Birth	Specimen No.	Sex	Estimated Age (days) from Birth when Sampled
1 30.v.54	3	?	1
	4	♂	4, 11, 18
2 16.vii.54	5†	♀	6, 20, 34, 48, 62, 76, 83, 97, 125, 137
	6†	♂	6, 20, 34, 48, 62, 76, 83, 97, 125, 153, 181, 209, 265, 321, 377
3 14.x.54	8	♀	21
	9	♂	21
	10	♂	21
	11	♂	21
4 25.xi.54	12	♀	13, 20, 27
5 19.xi.54	16	♀	34
	17	♂	34
	18	♂	34
6 18.xii.54	20	♀	40, 45
	22†	♀	54, 61, 75, 110, 166, 222, 463, 526
	23	♂	40
7 3.vii.55	24‡	?	2
8 6.vii.55	27	♀	27
9 6.xii.55	28†	?	7
	29	?	15
	30	♀	23

* Advanced intra-uterine specimen not included.

† Both skin and hair sampled from 48 days onward.

‡ Complete serial sections prepared.

of skin from each sample were sectioned serially at 8 μ , one parallel to the skin surface, and the other to the long axes of the hair follicles. All the sections were stained in haemalum, eosin, and picric acid. Some unstained whole mounts of skin were also prepared.

(d) Terminology

A new terminology for pelage hair follicles in *P. nasuta*, based on one proposed by Hardy and Lyne (1956a) for wool follicles in sheep, is used in preference to that adopted by Gibbs (1938) or Hardy (1947).

Primary follicles (P) and *secondary follicles (S)* are recognized. The first-formed follicles are termed *original central primary follicles (PCO)*. A *PCO* follicle occupies the central position of a follicle group and it is readily distinguished during development by the presence of a simple sudoriferous gland. Two later-formed follicles which usually appear simultaneously on either side of the *PCO* follicle are termed *original lateral primary follicles (PLO)*. A sudoriferous gland is sometimes, but not always, formed in association with a *PLO* follicle. After the formation of this trio group the *original secondary follicles (SO)* develop, initially between the *PCO* and *PLO* follicles on the ental side of the group. No sudoriferous glands are formed in association with the *SO* follicles. All original follicles arise directly from the epidermis and have independent orifices on the skin surface.

Before the end of the first hair growth cycle a new generation of follicles develops by branching from the *PLO* and *SO* follicles. Those which arise from the *PLO* follicles are termed *derived lateral primary follicles (PLD)* and those which arise from the *SO* follicles are termed *derived secondary follicles (SD)*. During the second and later cycles of hair growth more *PLD* and *SD* follicles develop from the *PLO* or *PLD* follicles or both, and from the *SO* or *SD* follicles or both. Follicles which arise from the *PCO* follicles are termed *derived central primary follicles (PCD)*. All these derived follicles arise by branching from existing follicles and share a common orifice with their parent.

The terms for the various components of the follicle group are similar to those used by Hardy and Lyne (1956c).

III. THE DEVELOPMENT OF INDIVIDUAL FOLLICLES

(a) Stages of Follicle Development

The eight fundamental stages in hair follicle development for mammals in general (Hardy and Lyne 1956b, 1956c) can be recognized in both *P* and *S* follicles in *P. nasuta*.

(i) *Original Central Primary Follicles*.—See Figure 1. The various stages are:

Stage F1: Follicle plug.—A plug of cells from the epidermis extends into the dermis. An aggregation of dermal cells appears beneath the epidermal plug (Plate 2, Fig. 1).

Stage F2: Pre-papilla.—The base of the epidermal plug flattens prior to invagination when it is still relatively short; the length of the plug does not usually exceed its diameter, and is sometimes considerably less (Plate 2, Fig. 2).

Stage F3: Papilla.—The base of the plug becomes recognizably concave and it can be subdivided according to the shape of the dermal papilla and the development of the associated glands. At stage *F3a* (Plate 2, Fig. 3) the base of the epidermal plug becomes recognizably concave, the length of the follicle being less than its diameter. At stage *F3b* (Plate 2, Fig. 4) the length of the follicle is usually slightly

more than its diameter and the sudoriferous gland rudiment appears as a solid plug from the ental side of the follicle. The length of the dermal papilla is less than its diameter. At stage F3c (Plate 2, Fig. 5) the length of the follicle is more than twice its greatest diameter and the length of the papilla is equal to or greater than its diameter. A bilobed sebaceous gland with differentiated cells is formed below the junction of the sudoriferous gland with the follicle. Further development of the sudoriferous gland has taken place and it may show a distinct swelling.

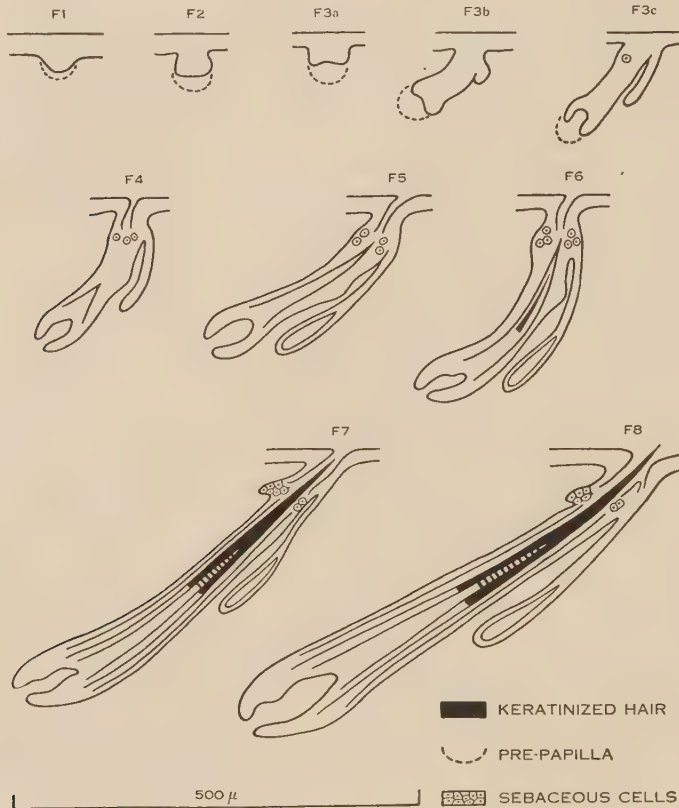


Fig. 1.—Diagram from camera lucida drawings of stages F1–F8 in development of original central primary pelage hair follicles in *P. nasuta*.

Stage F4: Hair cone.—The hair cone is formed by the cells of Henle's layer of the inner root sheath (Plate 2, Fig. 6). The length of the follicle is approximately three to four times its greatest width. A hair canal containing keratinized cells is formed and it opens at the skin surface. An ental swelling of the outer root sheath is usually formed and the sudoriferous gland may contain a small lumen.

Stage F5: Advanced hair cone.—The tip of the hair cone is refractile and it reaches the level of the base of the sebaceous gland (Plate 2, Fig. 7). Inside the hair cone Huxley's layer and the developing hair cuticle and cortex can be recognized. Some follicles still have a distinct ental swelling of the outer root sheath.

Stage F6: Hair formation.—The tip of a keratinized hair appears inside the hair cone which is solid and refractile, and lies at or above the level at which the sebaceous gland opens. All the follicle layers can be recognized and a lumen is formed within every sudoriferous gland.

Stage F7: Hair in epidermis.—The tip of the hair has emerged through the inner root sheath layers and lies at the epidermal level in the hair canal on the ental side of the follicle (Plate 3, Fig. 1). The inner root sheath layers split and fragment as they reach the hair canal. No ental swelling of the outer root sheath can be recognized.

Stage F8: Hair emerged.—The tip of the hair has penetrated the superficial layers of the epidermis.

(ii) *Original Lateral Primary Follicles.*—Stages *F1* to *F3a* are similar to those of *PCO* follicles except that the follicle is usually more elongated at *F2* and *F3a*. A sudoriferous gland is not usually formed in association with a *PLO* follicle, but when it is formed, stage *F3b* is recognized. At stage *F3c* the rudiment of the sebaceous gland, with differentiated sebaceous cells, is formed; the length of the dermal papilla is equal to or greater than its diameter. Stages *F4* to *F8* are similar in most respects to those for *PCO* follicles. No ental swelling of the outer root sheath has been observed in association with any *PLO* follicles.

(iii) *Original Secondary Follicles.*—The *SO* follicles go through the same stages of development as the *PLO* follicles except that they are usually more elongated and a sudoriferous gland is not formed at any stage.

(iv) *Derived Lateral Primary and Derived Secondary Follicles.*—See Figure 2 and Plate 4. The *PLD* and *SD* follicles go through the same stages of development as the original ones, except that:

- (1) Stage *F1* is a budding from a *PLO*, *PLD*, *SO*, or *SD* follicle at a level immediately below that at which the sebaceous gland opens, and not from the epidermis.
- (2) No additional sudoriferous or sebaceous gland is formed.
- (3) The hair canal, or hair funnel, into which the *PLD* or *SD* hair enters at stage *F7* is the common canal for the follicle bundle which was formed during the development of the *PLO* or *SO* follicle.

(v) *Derived Central Primary Follicles.*—*PCD* follicles are uncommon, and as they do not arise until after the first *PLD* and *SD* follicles are formed, only limited observations on their development have been possible.

A unique feature of the branching of *PCO* follicles, and some *PLO* follicles, is that the type of hair which develops within the derived follicle is different in size and form from that which develops within the parent follicle (Fig. 7). All the *PCO* follicles, and some *PLO* follicles, develop only large grooved hairs, whereas the *PCD* and *PLD* follicles develop only small circular hairs. In all skin samples examined the branching of the *PCO* follicle is at a level immediately below that at which the sebaceous gland opens.

(b) *The Elongation of Original Central Primary Follicles*

As most of the first generation *PCO* follicles are straight or only slightly bent it has been possible to measure them at different stages of development. Measure-

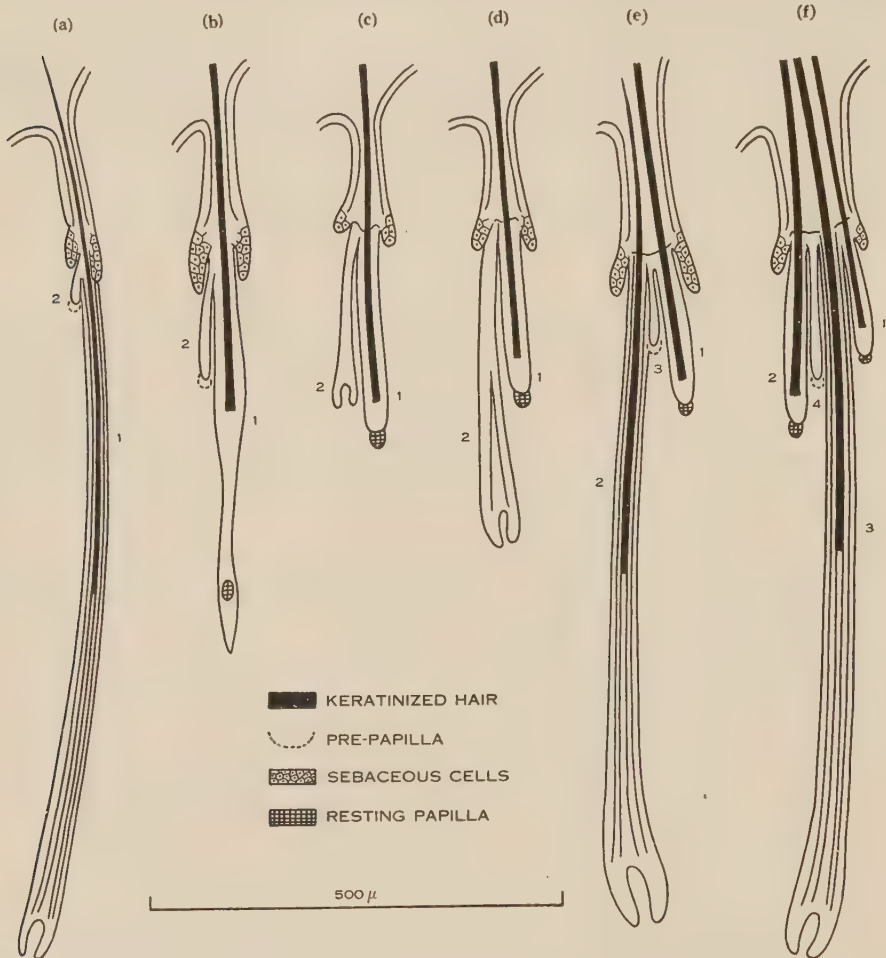


Fig. 2.—Diagram from camera lucida drawings of some of the stages in development of a bundle of lateral primary or secondary pelage hair follicles in *P. nasuta*. (a) Original follicle (1) at stage *F8* and first derived follicle (2) at stage *F1*. (b) Original follicle (1) with degenerating hair bulb, hair club, and resting papilla. First derived follicle (2) at stage *F1*. (c) Original follicle (1) with hair club and resting papilla. First derived follicle (2) at stage *F3c*. (d) First derived follicle (2) at stage *F4*. (e) First derived follicle (2) at stage *F8*, and second derived follicle (3) at stage *F1*. (f) First derived follicle (2) with hair club and resting papilla. Second derived follicle (3) beyond stage *F8* and third derived follicle (4) at stage *F1*. Follicle regeneration from the cells between the hair club and surviving cells of the papilla has not been observed.

ments of follicle length (i.e. the length along the long axis of the follicle, or in a straight line if the follicle is bent, from the epidermal-dermal junction to the base of the bulb) have been made with an ocular micrometer on serial sections cut parallel to the long axes of the follicles. The relation between the estimated age and the

length of the first generation *PCO* follicles up to 48 days is shown in Figure 3. Only the most advanced stage *F1* follicles have been measured.

The length of the follicle increases very rapidly after the emergence of the hair. At an estimated age of 40 days, when the most advanced *PCO* follicles are at stage *F8*, the follicle length is approximately 0.55 mm. Eight days later the *PCO* follicles have increased in length to about 2.0 mm, and the *PCO* hairs have grown

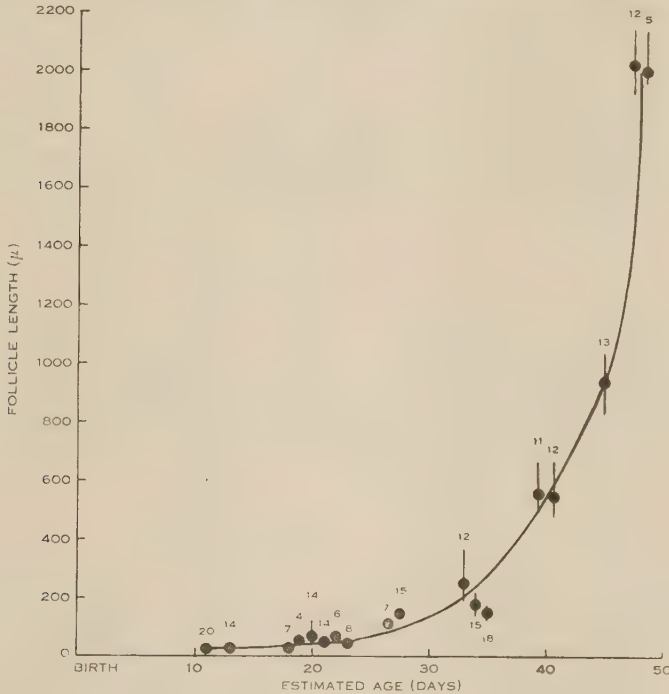


Fig. 3.—Relation between the estimated age and the length of the first generation original central primary follicles in *P. nasuta*. The number of follicles measured and the range of follicle length is shown when it falls outside the plotted mean. The curve through the points has been drawn by free-hand.

to a length of approximately 2.0 mm above the epidermis. No further elongation of the first generation *PCO* follicles has been observed although the hairs in these follicles increase to a final length of approximately 15.0 mm above the epidermis. The upper and lower parts of a fully developed *PCO* follicle at 48 days are illustrated in Plate 3, Figures 2, 3, and 4.

(c) Rate of Development of Follicles

The relation between the estimated age and the most advanced follicle stage found among the different types of follicles of the first hair cycle is shown in Figure 4. The *PCO* follicles begin their development on the midside between the 7th and 11th day after birth and reach stage *F8* in 29–33 days. The *PLO* follicles begin 23–27 days after birth and reach *F8* in 18–22 days, and the *SO* follicles, which begin at 27–34 days, reach *F8* in 14–21 days.

The first derived follicles begin their development while the follicles of the first hair cycle are still active. A few *PLD* follicles have been observed at 45 days, and at 48 days most of the *PLO* and *SO* follicles have derived follicles associated with them (Plate 5, Fig. 4). These derived follicles are at first relatively very small; they remain slender and appear to remain stationary at stage *F2* as long as their parent follicles are still actively growing. At 54, 61, and 62 days the most advanced derived follicles are still at stage *F2*. At 75 days the most advanced derived follicles

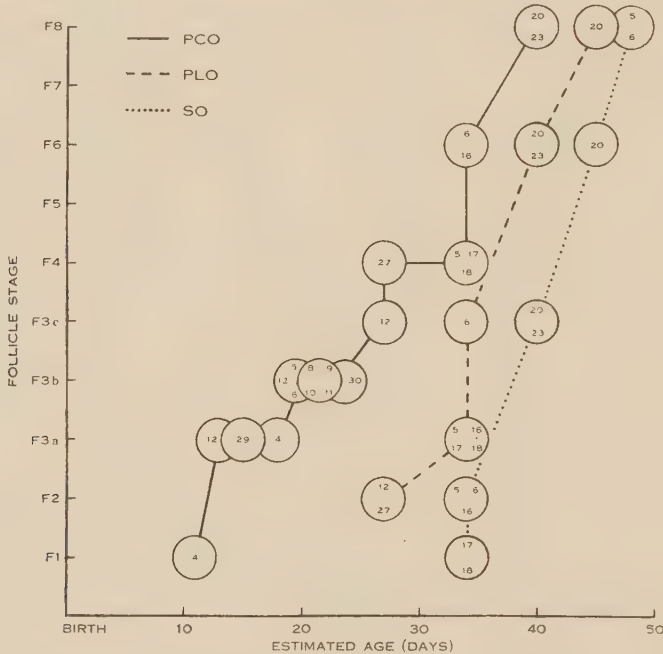


Fig. 4.—Relation between the estimated age and the most advanced follicle stage found among the different types of follicles of the first hair cycle in *P. nasuta*. The specimen numbers (see Table 1) are shown in the circles.

are at *F4*, and at 76 days one specimen has derived follicles at *F6* and its litter mate has derived follicles at *F8*. From these observations it was estimated that the first *PLD* and *SD* follicles developed from stage *F1* to stage *F8* in approximately 28–31 days. However, if the stationary phase is omitted, the rate of development of the first derived follicles is similar to that for the first *SO* follicles.

The first *PCD* follicle discovered was at an early stage of development in a specimen (No. 5) at 83 days. The same specimen at 97 days has some *PCD* follicles beyond stage *F8* (Fig. 7).

(d) *The Relation between the Follicle Types in the Skin and the Hair Types in the Coat*

Two main types of hair are readily distinguished in the coat of *P. nasuta*. The *PCO* follicles always produce coarse flattened guard hairs (Figs. 5(f), 5(g), and 5(i);

Plate 5, Fig. 4) which are grooved on the ectal side along the greater part of the shaft. These characteristic guard hairs are similar to the protective hairs described by Lyne and McMahon (1951) in the bandicoots *I. obesulus* and *P. gunnii*. The *S*

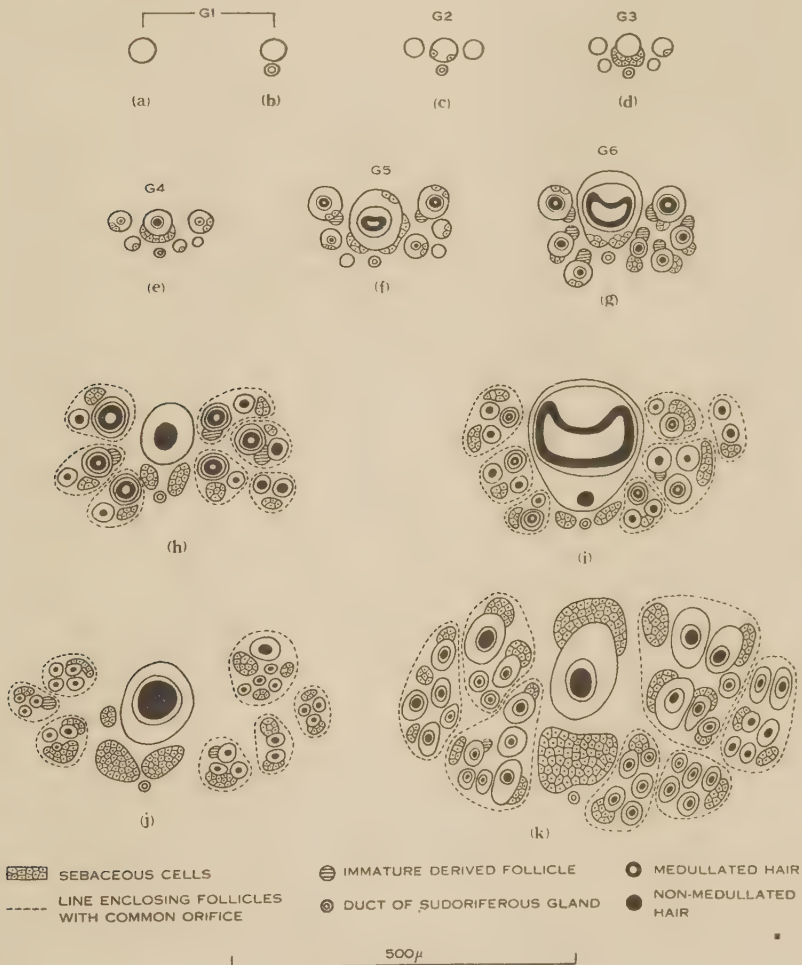


Fig. 5.—Diagram from camera lucida drawings of stages in development of the follicle group in *P. nasuta*. The sections are transverse to the follicles at the level of the sebaceous glands. (a)–(g) Stages G1–G6 of the first hair cycle. Stage G1 begins when the first original central primary follicles are at stage F1 at about 11 days after birth. Stage G6 is reached when the first original secondary follicles have emerging hairs at about 48 days of age. (h)–(k) The follicle group at approximately 83, 97, 125, and 377 days after birth. Dotted lines enclose the bundles of closely associated lateral primary and secondary follicles which have a common orifice.

follicles always produce fur hairs which are fine and circular in cross section. Some grooved hairs, usually smaller than those produced by the PCO follicles, are produced by the PLO follicles. All the hairs are medullated, except at the tip and base.

IV. THE DEVELOPMENT OF FOLLICLE GROUPS

(a) *Stages in the Development of the Follicle Group of the First Hair Cycle*

The main events in the development of the follicle group of the first hair cycle (Figs. 5(a)–5(g); Plate 5, Figs. 1, 2, 3, and 4) may be described by a series of stages prefixed by the letter *G* to distinguish them from the *F* stages of individual follicles. The stage numbers do *not* correspond with those proposed by Carter (1943) for the Merino and redefined by Hardy and Lyne (1956c).

Stage G1: PCO follicles present.—These follicles, which are more or less equally spaced on the skin surface, do not all develop at the same time. It is convenient to subdivide this stage. At stage *G1a* (Fig. 5(a)) the follicles are at stage *F1* to *F3a*. At stage *G1b* (Fig. 5(b)) the most advanced follicles are at stage *F3b*.

Stage G2: Trio groups formed by the addition of a PLO follicle on each side of some of the PCO follicles (Fig. 5(c)).—The most advanced *PCO* follicles are at stage *F3c*.

Stage G3: SO follicle formation begun (Fig. 5(d)).—The first plugs (*F1*) of *SO* follicles appear between the *PCO* and *PLO* follicles on the ental side of the group (Plate 5, Fig. 3). *PCO* follicles are at stage *F4* and *PLO* follicles are at *F3a*.

Stage G4: PCO hairs emerged (Fig. 5(e)).—Some *PCO* follicles are at *F8*. Some *PLO* follicles are at *F6* and some *SO* follicles are at *F3c*.

Stage G5: PLO hairs emerged (Fig. 5(f)).—Some *PLO* follicles are at *F8* and some *SO* follicles are at *F6*.

Stage G6: SO hairs emerged (Fig. 5(g)).—Some *SO* follicles are at *F8*.

(b) *The Development of the Follicle Group of the Second and Later Hair Cycles*

At stage *G5* the first *PLD* follicles appear immediately below the level at which the sebaceous gland opens, usually on the side of the *PLO* follicle nearest to the *PCO* follicle (Fig. 5(f)). At stage *G6* the first *SD* follicles appear at the same level as the *PLD* follicles, usually on the side of the *SO* follicle nearest to the *PCO* follicle (Fig. 5(g); Plate 5, Fig. 4). At this stage (*G6*) the first generation *PCO* follicles and most of the *PLO* follicles cease to increase in length. The most advanced *PLD* and *SD* follicles are at *F2*, and appear to remain stationary until the growth of the follicles of the first hair cycle ceases, and hair clubs are being formed. The hair canal into which the *PLD* or *SD* hair enters at stage *F7* is the common canal for the follicle bundle which was formed during the development of the *PLO* or *SO* follicle. When the first *PLD* and *SD* follicles reach stage *F8*, most of the *PLO* and *SO* hairs are still present as hair clubs (Figs. 2(e) and 2(f)).

Only an occasional *PCO* follicle gives rise to *PCD* follicles. There is, however, a normal replacement of *PCO* hairs as shown in Figure 6, the new hairs developing on the ectal, or concave side, of the old hairs.

Before the end of the second and later hair cycles, more *PLD* and *SD* follicles arise by branching from the *PLO* or *PLD* follicles or both and from the *SO* or *SD* follicles or both (Figs. 2(e) and 2(f)). When the second generation of derived follicles reaches stage *F8* the follicle bundle is usually composed of three closely associated follicles sharing a common orifice.

(c) *The Size of the Follicle Group*

Observations on the size of the follicle group at different ages have been made on the serial sections cut parallel to the skin surface. Counts have been made of the number of: original follicles in each group during the first hair cycle, orifices per

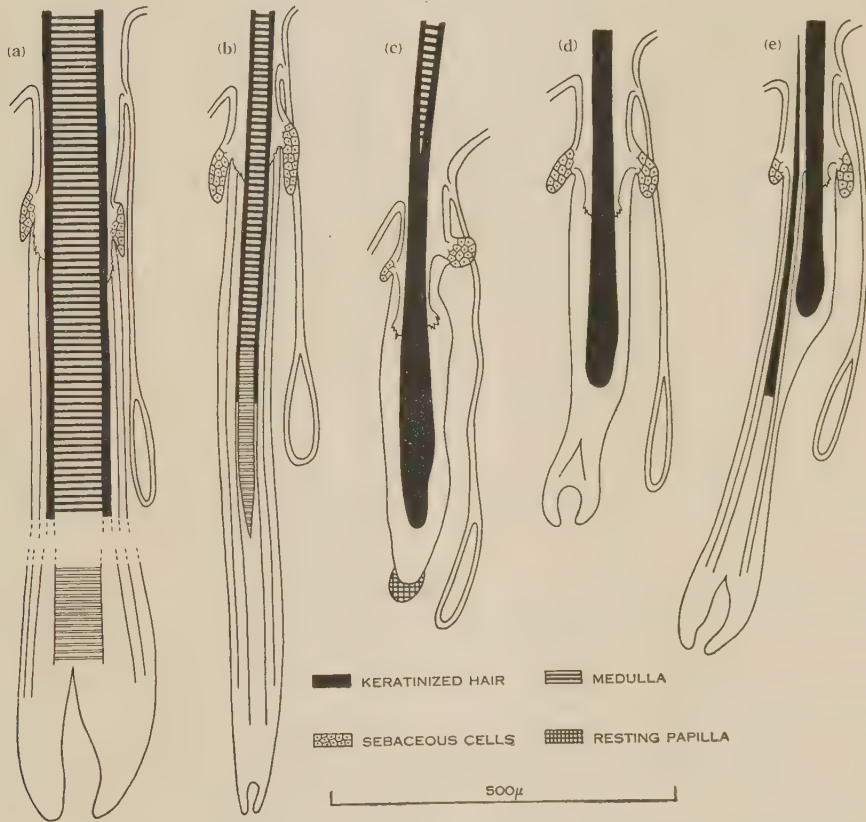


Fig. 6.—Diagram from camera lucida drawings of some of the changes in the original central primary follicles during a hair growth cycle in *P. nasuta*. (a) Upper and lower parts of a fully developed follicle. The follicle is at its maximum length of approximately 2.0 mm. (b) Beginning of resting phase. The hair has become thin at the base and its growth has almost ceased. A non-medullated hair club is being formed and the follicle bulb has decreased in size. (c) Resting phase. The follicle has shortened and a hair club is formed. The former dermal papilla cells are clustered at the base of the epidermal sheath surrounding the hair club. (d) A new follicle root, formed by proliferation of cells at the base of the resting follicle, has reached a stage equivalent to stage *F4* of the first hair cycle. (e) The new hair has emerged on the ectal (grooved) side of the old hair.

group, hairs per group, and hairs per orifice in the second and subsequent cycles. Summaries of the observations on the size of the follicle group from 34 to 45 days, and from 48 to 526 days are shown in Tables 2 and 3 respectively. These periods cover most of the first hair cycle, and the second and subsequent cycles respectively.

The counts of the number of original follicles per group (Table 2) reveal that in three out of the five specimens at 34 days, some of the groups contain only two

follicles; that is, some of the *PLO* and *SO* follicles have not appeared. * Also, the mean number of follicles per group is lower for these three specimens (Nos. 16, 17, 18; litter mates) than it is for the other specimens (Nos. 5, 6; also litter mates) at the

TABLE 2
SUMMARY OF OBSERVATIONS ON THE SIZE OF THE FOLLICLE GROUP FROM 34 TO 45 DAYS

Estimated Age (days)	Specimen No.	No. of Follicle Groups Examined	No. of Original Follicles per Group	
			Range	Mean
34	5	55	3-14	6.6
34	6	29	3-9	5.7
34	16	37	2-9	4.5
34	17	28	2-6	3.9
34	18	30	2-7	3.6
40	20	39	3-14	6.9
40	23	42	4-13	8.0
45	20	33	3-8	5.4

same estimated age. Although one specimen (No. 5) at 34 days has a few larger follicle groups and a higher mean number of original follicles per group than its

TABLE 3
SUMMARY OF OBSERVATIONS ON THE SIZE OF THE FOLLICLE GROUP FROM 48 TO 526 DAYS

Estim- ated Age (days)	Speci- men No.	No. of Follicle Groups Exam- ined	No. of Orifices per Group*		Total No. of Hairs per Group		No. of <i>PC</i> Hairs per Orifice		No. of <i>PL</i> and <i>S</i> Hairs per Orifice	
			Range	Mean	Range	Mean	Range	Mean	Range	Mean
48	5	94	4-11	6.3	4-11	6.3	0	1.0	0	1.0
48	6	26	4-11	6.9	4-11	6.9	0	1.0	0	1.0
97	5	21	3-10	5.7	7-30	15.9	1-4	2.1	0-6	3.0
110	22	14	4-8	6.4	9-23	15.3	0	2.0	1-4	2.5
125	5	32	3-10	5.9	8-27	15.0	1-4	1.8	0-6	2.7
153	6	14	4-9	5.9	15-40	22.9	1-4	1.4	1-8	4.4
166	22	10	3-7	4.8	11-27	16.9	1-2	1.1	2-7	4.2
222	22	16	3-9	5.8	8-27	16.4	1-3	1.7	1-6	3.1
265	6	19	3-7	4.3	7-37	19.8	1-6	2.4	1-8	5.3
377	6	7	4-9	6.6	19-49	34.1	1-3	1.6	0-11	5.8
463	22	10	4-11	6.5	14-33	22.0	1-2	1.4	1-7	3.8
526	22	27	3-9	6.0	13-36	24.1	1-3	1.3	1-10	4.6

* Equals the number of original follicles per group.

litter mate (No. 6), the most advanced *PCO* and *PLO* follicles in the latter specimen are at a later stage of development than the same follicle types in the former specimen (Fig. 4). At 40 days both specimens (litter mates) have several large follicle groups

and the mean number of original follicles per group is especially large in one specimen. If the number of original follicles per group from 34 to 45 days (Table 2) is compared with the number of orifices per group from 48 to 526 days (Table 3), it is found that, with the exception of three specimens (Nos. 16, 17, 18) at 34 days, there are very

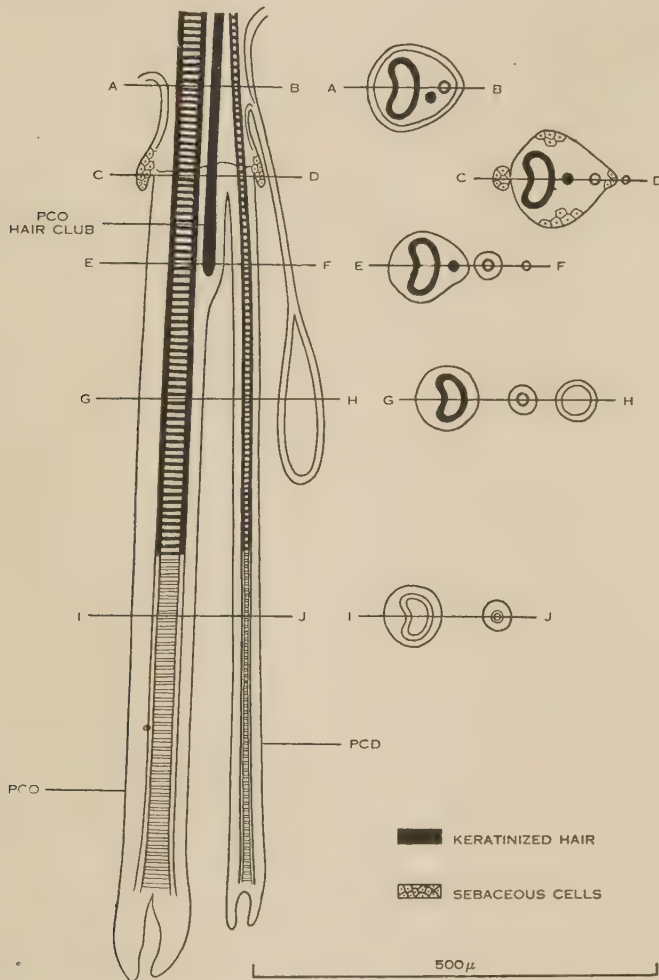


Fig. 7.—Diagram from camera lucida drawings of a bundle of central primary pelage hair follicles in *P. nasuta*. Specimen 97 days old. Longitudinal section on left is drawn from reconstruction of transverse sections, several of which are shown on the right. The first generation *PCO* hair is still present as a hair club. Note that the *PCD* hair differs in size and form from the new *PCO* hair.

few differences between the two sets of figures. These counts confirm other observations that only the follicles of the first hair cycle arise from the epidermis. The number of orifices per group, which equals the number of original follicles per group, remains constant after all the follicles of the first hair cycle have been formed at about 40 days.

Three specimens, one at 34 days and two at 40 days, have a few groups containing up to 13 or 14 original follicles (Table 2). In contrast, the number of orifices per group from 48 to 526 days does not exceed 11 (Table 3). This suggests that the larger groups at 34 and 40 days, which usually have a sudoriferous gland associated with one or even both *PLO* follicles, later separate into two (possibly three) distinct groups. If this occurs it is thought that a *PLO* follicle becomes the central member of the new follicle group. This *PLO* follicle would then be classified as a *PCO* follicle. It is possible that the few *PCO* follicles which branch (Fig. 7) were initially *PLO* follicles but, with the present material, there does not seem to be any way of testing this hypothesis.

With only one exception, not more than two *PCD* follicles in a *PC* bundle, and not more than four *PC* hairs per orifice, have been observed. The exceptional *PC* orifice has six hairs (Table 3) but no sudoriferous gland is discernible. This is further evidence that a few *PLO* follicles later occupy the central position of a group.

The figures for the number of *PL* and *S* hairs per orifice (Table 3) show that, from 97 to 526 days, bundles with 2, 3, 4, or 5 hairs are common. The largest bundle examined contains 11 follicles with emerging hairs.

(d) *The Density of the Follicles*

Only very limited studies have been made on the density of the hair follicles because the skin samples collected were of various sizes and no corrections were made for changes in area at collection or during histological preparation.

Counts were made of the *PC* and *PL* plus *S* follicles (immature derived follicles not included) occurring in sections of skin of areas of 1–4 mm², cut parallel to the surface and at the sebaceous gland level. Two specimens (Nos. 5, 22) were examined at estimated ages ranging from 48 to 526 days. At 48, 97, 125, 166, 222, and 526 days the approximate number of *PC* follicles per mm² was 26, 21, 17, 11, 13, and 8 respectively, and, in the same specimens at the same ages, the approximate number of *PL* plus *S* follicles per mm² was 130, 152, 161, 163, 107, and 140 respectively. As the number of original follicles is complete before 48 days, and there is little branching from the *PCO* follicles, it seems probable that the changes in density of the *PC* follicles merely reflect the normal skin expansion accompanying growth.

V. DISCUSSION

Histological study of the development and replacement of pelage hairs in *P. nasuta* has revealed several unique features not previously described in any mammal.

In the development of the *PCO* follicles of *P. nasuta* the pre-papilla stage (*F2*) and the first invagination (*F3a*) occur when the follicle is still relatively very short and before the first appearance of the sudoriferous gland rudiment. In *T. vulpecula*, Gibbs (1938) figures the formation of a sudoriferous gland rudiment prior to the invagination of the base of the follicle. In the central and lateral primary follicles of sheep, described by Hardy and Lyne (1956c), the sudoriferous gland rudiment appears at stage *F2*, and the follicle is considerably more elongated than it is in *P. nasuta* at stage *F3a*.

In *P. nasuta*, a sudoriferous gland may develop in association with one or both *PLO* follicles but a typical group has only one sudoriferous gland associated with the *PCO* follicle. Hardy (1947) described sudoriferous glands associated with lateral primary follicles in one of three specimens of *P. nasuta* which she examined. In the various breeds of sheep examined by Carter (1955) a sudoriferous gland was always formed in association with the lateral primary as well as the central primary follicles.

The *SO* follicles in *P. nasuta*, which develop on the ental side of the group, may be compared with the *SO* follicles in sheep (Hardy and Lyne 1956c). In sheep, the first-formed *SO* follicles develop on the ectal side of the group.

The *PCO* follicles in *P. nasuta* develop from stage *F*1 to stage *F*8 in about 29–33 days while the equivalent follicles in the mouse (Hardy 1949) and the sheep (Hardy and Lyne 1956c) pass through the same stages in about 9 and 38 days respectively. In *P. nasuta*, the *PLO* and *SO* follicles, particularly the latter, develop at a faster rate than the equivalent follicles in the sheep. The *SO* follicles in *P. nasuta* develop from *F*1 to *F*8 in about 14–21 days whereas the *SO* follicles in the sheep pass through the same stages in about 40 days. This suggests that the branching of the *SO* follicles, which begins at a much earlier stage in the sheep than in *P. nasuta*, has considerable influence on the rate of development of these follicles.

The replacement of the *PCO* follicles in *P. nasuta* is similar in most respects to that which has been described by Segall (1918) for the guinea pig, and by Dry (1926) for the mouse. The method of replacement of the *PLO* and *SO* follicles in *P. nasuta* apparently has not been previously described in mammals. There is, however, some evidence to suggest that it may be a common one. In the marsupial mole, *Notoryctes typhlops*, the hairs are arranged in bundles usually containing from 11 to 19 hairs in separate root sheaths (Sweet 1907). The follicles of each bundle have a common neck and a common orifice at the skin surface. The development of these bundles was not described but it seems probable that follicle branching occurs in this marsupial. In the adult marsupial material examined by Hardy (1947) the typical follicle group consisted of one large central hair follicle flanked by from two to eight lateral bundles of smaller follicles, each bundle with a common follicle opening at the skin surface. Branching follicles are not described by Hardy (1947) but in her Plate V, Figure 12, illustrating a follicle group from the bandicoot, *Isoodon torosus* Ramsay, she shows several immature follicles which are probably derived follicles similar to those described in the present study.

In the branching secondary wool follicles of sheep, described by Hardy and Lyne (1956c), the branching is usually at or above the level of the sebaceous glands and it begins at a much earlier stage in the development of the *SO* follicles than in *P. nasuta*. Also, the type of follicle branching in sheep is associated with the maturation of the follicle group and not with the replacement of hair as it is in *P. nasuta*. No branching primary follicles have been described in sheep.

According to Weddell and Pallie (1955) as many as seven separate hairs, derived from closely related hair follicles, may emerge from a single small orifice in the skin of the rabbit ear. The development of these bundles of follicles is not described but the observations suggest that follicle branching occurs in this mammal.

In the adult chinchilla Wilcox (1950) described bundles with as many as 75 hairs emerging from a single orifice; this would suggest that branching occurs in this species. In the mouse, Dry (1926) says that bundles of two, three, or four hairs are grown, one by one, in the same follicle. However, he describes two exceptional bundles in the tail of an animal more than a year old; each bundle had two hairs in separate root sheaths below the persistent part of the follicle. In the platypus (*Ornithorhynchus*) the adult follicle group consists of a central follicle surrounded by a number of bundles of follicles (Poulton 1894); each bundle was originally a simple follicle from which one fibre projected. Poulton says that the bundles of follicles are formed by branching from some part of the first-formed follicles, and sebaceous glands are situated at the level at which the follicles unite to form a common follicular neck. Spencer and Sweet (1899) examined hair development in both the spiny ant-eater (*Tachyglossus*) and the platypus and they confirmed Poulton's observations on the branching of follicles in the latter.

VI. ACKNOWLEDGMENTS

The author is indebted to Miss M. J. Heideman and Miss A. J. Bathgate for assistance with the figures and histological preparation, and to Mr. I. T. Roper for photography.

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EXPLANATION OF PLATES 1–5

All figures (except Plate 1) are of sections through skin from the mid-lateral region of the trunk of the bandicoot, *P. nasuta*. The ages of all specimens are estimated. The staining is with haemalum, eosin, and picric acid. *C*, stratum corneum; *Co*, cortex; *Cu*, cuticle of hair; *D*, derived lateral primary or derived secondary follicle; *DP*, dermal papilla; *ES*, ental swelling of outer root sheath; *G*, stratum germinativum; *H*, keratinized hair; *HC*, hair canal; *HF*, hair funnel; *HN*, hair cone; *M*, medulla; *O*, original lateral primary or original secondary follicle; *P*, pigment; *PCO*, original central primary follicle; *PLD*, derived lateral primary follicle; *PLO*, original lateral primary follicle; *PP*, pre-papilla; *RP*, resting papilla; *S*, stratum spinosum; *SCS*, space formed by disintegration of sebaceous cells; *SD*, derived secondary follicle; *SG*, sebaceous gland with differentiated cells; *SO*, original secondary follicle; *SudG*, sudoriferous gland; *SudGD*, sudoriferous gland duct; *SudGR*, sudoriferous gland rudiment.

PLATE 1

Perameles nasuta

- Fig. 1.—Specimen No. 5 at 34 days. Note that the specimen is naked except for vibrissae. The first hairs emerge on the mid-lateral region of the trunk at 40 days.
- Fig. 2.—The same specimen as shown in Plate 1, Figure 1, at 48 days. Note that the specimen is now completely covered with hair.
- Fig. 3.—Specimen No. 6 (litter mate of No. 5) at 321 days. The specimen is now almost fully grown.

PLATE 2

Longitudinal sections of original central primary follicles at stages *F1*–*F5*

- Fig. 1.—Two *PCO* follicles at stage *F1* in an 11-day-old specimen.
- Fig. 2.—Stage *F2* at 21 days.
- Fig. 3.—Stage *F3a* at 18 days.
- Fig. 4.—Stage *F3b* at 21 days.
- Fig. 5.—Stage *F3c* at 27 days.
- Fig. 6.—Stage *F4* at 34 days.
- Fig. 7.—Stage *F5* at 34 days. The tip of the hair cone is not clearly seen.

PLATE 3

Development of original central primary follicles

- Fig. 1.—Longitudinal section of a *PCO* follicle at stage *F7* in a specimen at 40 days.
- Fig. 2.—Longitudinal section of upper part of a fully developed *PCO* follicle in a specimen at 48 days.
- Fig. 3.—Longitudinal section of lower part of follicle shown in Plate 3, Figure 2.
- Fig. 4.—Almost transverse section of a fully developed *PCO* follicle at the level of the dermal papilla in a specimen at 48 days. The dermal papilla is grooved on the ectal side.

PLATE 4

Longitudinal sections of bundles of lateral primary or secondary follicles showing some stages of development

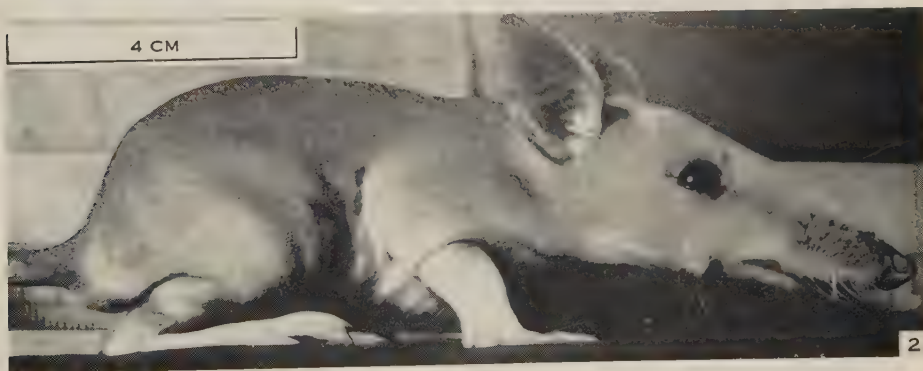
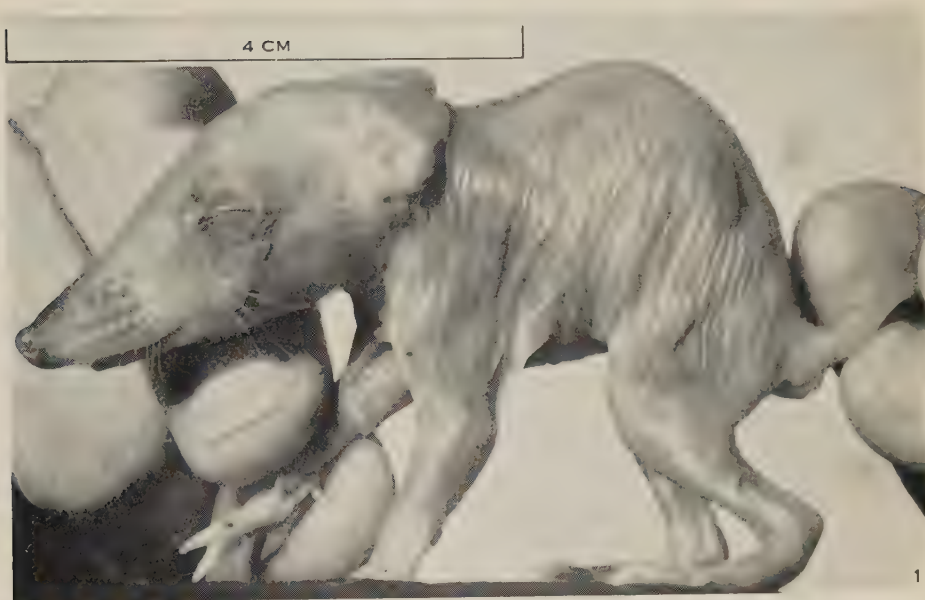
- Fig. 1.—Upper part of *SO* follicle at stage *F8* and *SD* follicle at stage *F1* in a specimen at 48 days.
- Fig. 2.—Part of *PLO* or *SO* follicle with hair club (not clearly seen) and derived follicle (*PLD* or *SD*) at stage *F1* in a specimen at 61 days.
- Fig. 3.—*PLO* or *SO* follicle with hair club and derived follicle at about stage *F3c* in a specimen at 75 days.
- Fig. 4.—New derived follicle with tip of pigmented hair at base of hair funnel and earlier formed follicle with hair club. Specimen at 153 days.
- Fig. 5.—Part of a bundle of three *PL* or *S* follicles in a specimen at 265 days. Active follicle in centre and resting follicles with hair clubs on either side.

PLATE 5

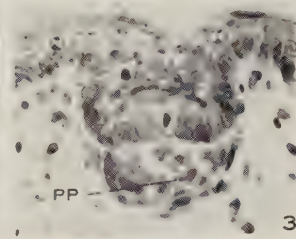
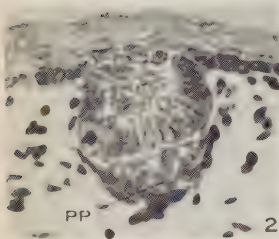
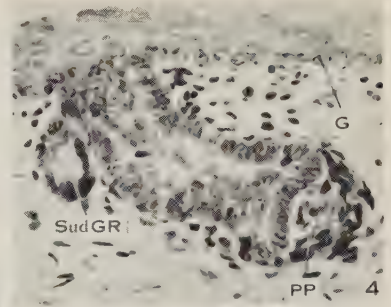
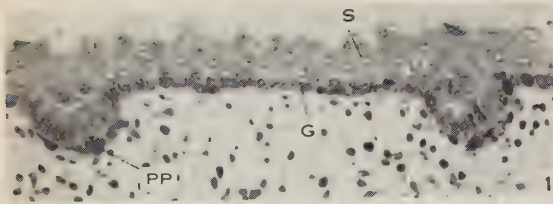
Transverse sections of follicle groups showing some stages of development

- Fig. 1.—Stage *G1a* at 20 days. *PCO* follicle at stage *F3a*.
- Fig. 2.—Stage *G2* at 27 days. *PCO* follicle at stage *F3b* and *PLO* follicles at stage *F2*.
- Fig. 3.—Stage *G3* at 34 days. *PCO* follicle at stage *F4*, *PLO* follicles at stage *F3a*, and *SO* follicles at stage *F1*.
- Fig. 4.—Stage *G6* at 48 days. *PCO*, *PLO*, and *SO* follicles at or beyond stage *F8*. *PLD* and *SD* follicles at *F1*–*F2*.
- Fig. 5.—Portion of a follicle group showing the *PCO* follicle and three complete bundles of follicles (one *PL* and two *S*) at a level between the epidermis and the sebaceous glands. Specimen 125 days old.
- Fig. 6.—Portion of follicle group shown in Plate 5, Figure 5, at a level below the sebaceous glands.
- Fig. 7.—A complete follicle group below the level at which the sebaceous glands open. Specimen 377 days old.

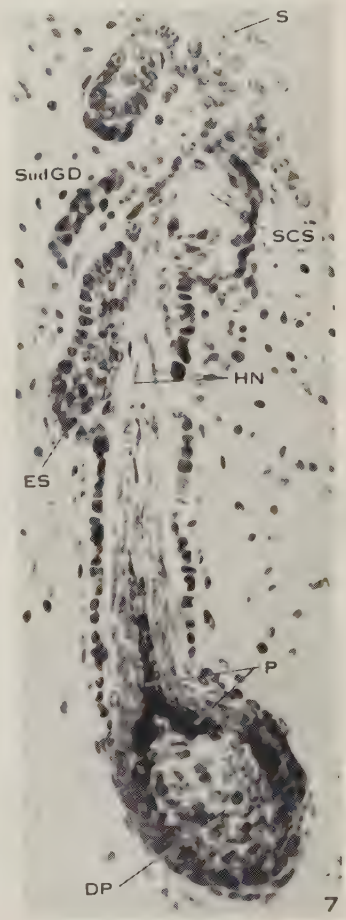
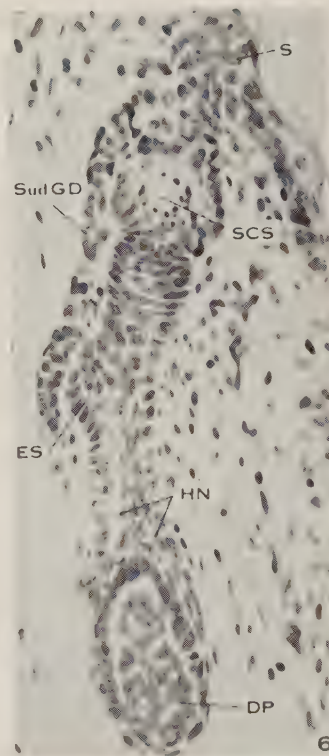
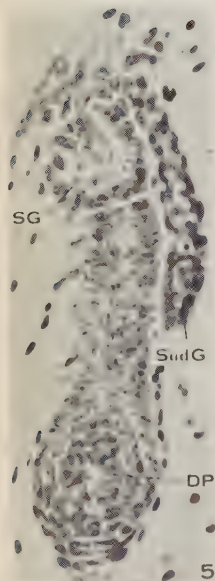
HAIR GROWTH IN THE BANDICOOT



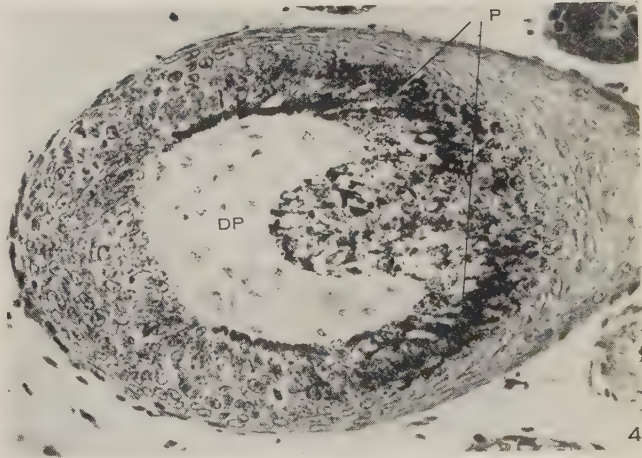
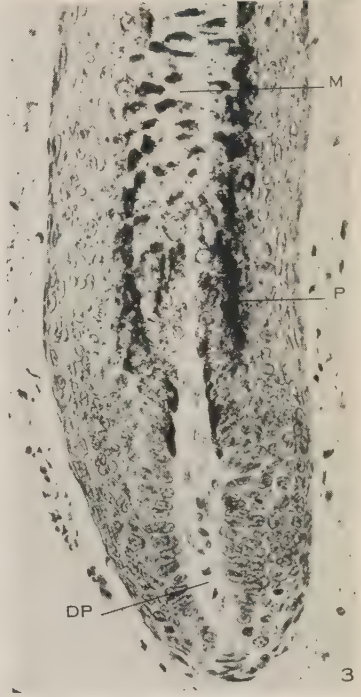
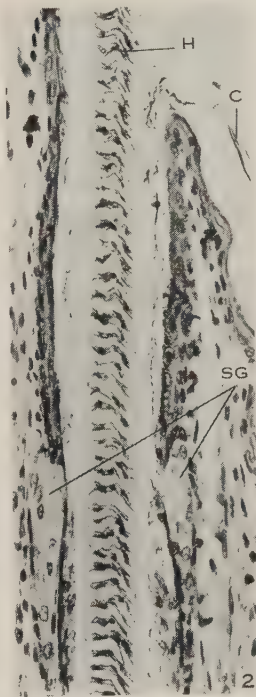
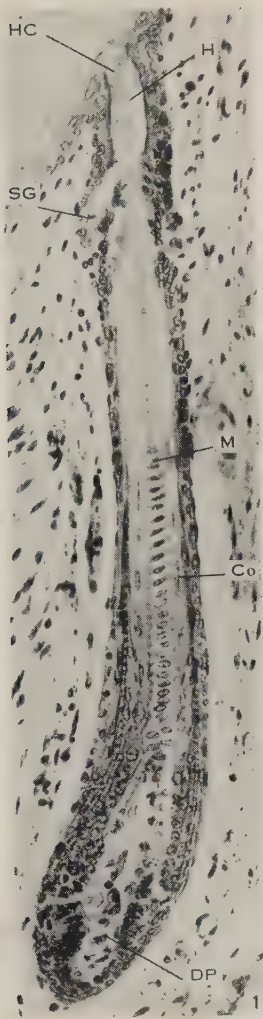
HAIR GROWTH IN THE BANDICOOT



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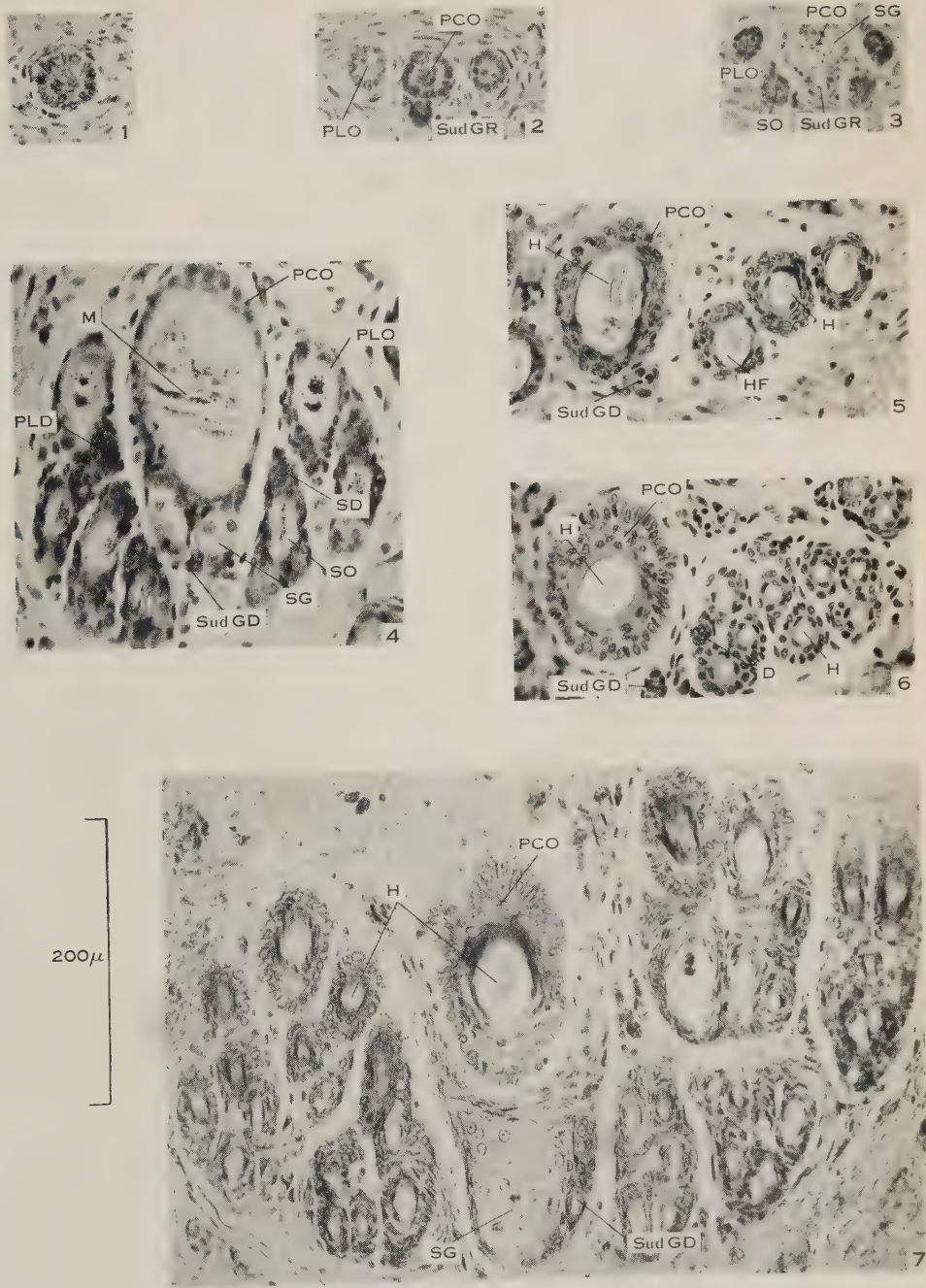
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HAIR GROWTH IN THE BANDICOOT



HAIR GROWTH IN THE BANDICOOT



METABOLISM OF SULPHUR AMINO ACIDS

I. THE UPTAKE OF CYSTEINE BY RAT LIVER

By EVA EDEN,* HELEN B. GRANOWSKI,* W. G. JONES,* and JUDITH A. LINNANE*

[*Manuscript received December 6, 1956*]

Summary

Cysteine was administered to rats by stomach tube or by intraperitoneal injection. In some animals the concentration of glutathione in the liver was first lowered to 10 per cent. of the normal level by injecting the animals with bromobenzene. When 30 mg of cysteine were given by stomach tube, 75 per cent. of the cysteine taken up by the liver in normal rats was oxidized in 1 hr to products other than cystine. In animals treated with bromobenzene all the cysteine was converted to glutathione and no increase could be found in the oxidized sulphur fraction. In both groups of animals only traces of cysteine were present.

The accumulation of free cysteine in the liver could only be obtained if the cysteine was given intraperitoneally and if very large doses (150 mg) were used.

I. INTRODUCTION

In normal rat livers, cysteine contributes less than 1 per cent. to the total free amino acid pool (Wu 1954), whereas its concentration in protein molecules is much higher (Block and Bolling 1951). Much of the cysteine taken up by the liver is used for protein synthesis, and some is built up into glutathione (Anderson and Mosher 1951). In addition, the liver contains enzyme systems capable of oxidizing the sulphhydryl group of cysteine to cystine and to other oxidation products (Singer and Kearney 1955).

In the present investigation, the conditions necessary for the accumulation of cysteine and the relative importance of the different metabolic pathways for the removal of cysteine were studied. It was found that cysteine conversion to glutathione could best be followed in animals where the concentration of glutathione in the liver had first been lowered. This is often done by feeding the rats a diet containing suboptimal amounts of sulphur amino acids (Barford and Eden 1956), but the glutathione concentration can be lowered more rapidly by administering a compound that will remove some of the cysteine present in the body. Halogenated hydrocarbons have been used for this purpose because they are detoxicated by conjugation with cysteine and the mercapturic acids thus formed are excreted in the urine (Koch-Weser *et al.* 1953). The effect of one of these compounds, bromobenzene, on the concentration of glutathione in the liver was investigated in some detail and the necessary conditions were established so that a single injection would lower the concentration of glutathione to 10 per cent. of its normal level. These animals were then used for the study of cysteine metabolism in the same manner as for normal rats.

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II. METHODS

(a) *Animals*

Female albino rats of 150–250 g body weight were used. Bromobenzene treatment consisted of intraperitoneal injection of 75 mg (0.05 ml) of bromobenzene/100 g body weight (for discussion of bromobenzene treatment see Section III). Both injected and normal animals were fasted for 16 hr to eliminate any absorption of remaining food from the intestine.

In the early experiments the animals were dosed with glucose, but as this caused no increased accumulation of sulphur-containing compounds in the liver it was omitted in the later experiments. Only when cysteine was given by stomach tube was glucose added and in these experiments 30 mg of cysteine hydrochloride and 150 mg of glucose were dissolved in 1 ml of water. Although this solution was acid it was not neutralized because of the acid pH of the stomach. When cysteine was injected intraperitoneally the solution was adjusted to approximately pH 4 with sodium bicarbonate.

In some cases repeated samples of liver were removed from the same animals. The rats were anaesthetized with "Nembutal" (5 mg/100 g of body weight), a trachea cannula was inserted, and artificial respiration was applied when necessary. The abdomen was opened, a sample of liver (approx. 0.3 g) removed, and the bleeding stopped with gelatin sponge. Then the animal was dosed with cysteine by stomach tube and further samples of the liver were removed at 30-min intervals. With this technique the animals could be kept alive for approximately 2 hr.

(b) *Chemical Methods*

Samples of liver (0.67 g) were homogenized in trichloroacetic acid (5 per cent.) and the homogenates were made up to 10 ml. Trichloroacetic acid proved unsatisfactory when the enzymatic method was used for the determination of glutathione, and for these estimations the homogenates were prepared in sulphosalicylic acid (2 per cent.).

Total sulphur was determined after the digestion of organic sulphur by adding barium chloride and measuring the turbidity thus obtained (Denis and Reed 1926). The specific brucine method was used for the determination of cysteine (Hird and Springell 1954).

The concentration of sulphydryl groups was estimated by the nitroprusside test (Grunert and Phillips 1951). Cystine was determined by reaction with a concentrated cyanide solution followed by estimation of the cysteine sulphydryl groups produced. The reaction was carried out by incubating 0.5 ml of the protein-free extract with 0.5 ml of a solution containing potassium cyanide (5 per cent.) in potassium carbonate (28 per cent.). The cyanide reaction yields approximately one thiol group for each disulphide and with each set of estimations a standard cystine solution was included from which the extent of reaction could be calculated. The amount of oxidized sulphur was calculated by subtracting the value obtained for sulphydryl sulphur from that of total sulphur.

Glutathione was determined by means of the specific glutathione reductase method. All sulphydryl groups were oxidized with hydrogen peroxide to the disulphide form, then glutathione reductase was added for the regeneration of glutathione. Other disulphide compounds are not reduced by this enzyme (Racker 1954). The actual procedure used was as follows: to 1.5 ml of tissue extract in 2 per cent. sulphosalicylic acid was added 0.2 ml of tris(hydroxymethyl)amino-methane buffer (0.5M, pH 7.4), 0.1 ml of disodium ethylenediaminetetra-acetate (5×10^{-3} M), and 0.1 ml of hydrogen peroxide (0.3M) and the tube kept at 37°C for 5 min. The solution was then adjusted to pH 7.4 with sodium hydroxide and the reduction system was added: triphosphopyridine nucleotide (0.1 ml, 3×10^{-4} M), the sodium salt of glucose-6-phosphate (0.1 ml, 6×10^{-2} M), and 0.5 ml of pea enzyme extract. This extract contained the glutathione reductase and also the glucose-6-phosphate dehydrogenase which was used to regenerate the triphosphopyridine nucleotide. The enzyme extract was prepared according to Mapson and Goddard (1951). The test system was made up to 2.7 ml with water and was incubated for 30 min at 37°C. The reaction was then stopped with 0.3 ml of 20 per cent. sulphosalicylic acid and an aliquot of the supernatant was used for the nitroprusside test. In normal tissue extracts the recoveries of added glutathione were always over 90 per cent.; however, large amounts of cysteine interfered with the determination of oxidized glutathione.

(c) Chromatography

Paper chromatography was used to confirm the results obtained by the chemical methods. Before the extracts were chromatographed the trichloroacetic acid was removed by extracting an aliquot (2 ml) twice with equal volumes of peroxide-free ether. The sulphydryl compounds were then coupled with *N*-ethyl maleimide (0.1 ml of a 0.05M solution) in order to prevent their oxidation during chromatography (Smith and Tuller 1955), the extracts were adjusted to pH 6 with sodium hydroxide and were dried overnight in a vacuum desiccator. The residue was dissolved in 0.2 ml of water and 30 μ l was spotted on to Whatman No. 1 paper. For most work unidimensional chromatography was satisfactory and the solvent used was generally butanol-acetic acid-water (4 : 1 : 1). The papers were sprayed either with ninhydrin (Williams and Kirby 1948), or with an azide-iodine solution for the detection of sulphur amino acids (Chargaff, Levine, and Green 1948).

III. RESULTS

(a) The Depletion of Liver Glutathione

In preliminary experiments, administration of bromobenzene was as described by Koch-Weser *et al.* (1953). The animals were fasted 24 hr before injection and then were injected intraperitoneally with 0.1 ml of bromobenzene/100 g body weight; they were killed 48 hr later. This treatment lowered the concentration of glutathione in the liver to about 10 per cent. of the normal level. A milder and simpler procedure proved to be equally satisfactory; the dose was halved and the initial period of fasting was eliminated. Table 1 shows that, with this treatment, after 16 hr the concentration of glutathione had decreased from 21 to 2 mg of

glutathione sulphur/100 g of liver and remained at this low level for at least another 8 hr. With increased length of time there was a gradual restoration of the glutathione concentration, presumably by the mobilization of the body stores of cysteine. The time of this recovery was variable, in some animals being complete by 48 hr. The

TABLE 1
CHANGES IN THE SULPHUR-CONTAINING COMPOUNDS OF THE LIVER
AFTER INJECTION WITH BROMOBENZENE

Each value represents the average of five animals. The rats were injected intraperitoneally with 0.05 ml of bromobenzene/100 g body weight

Time (hr)	Glutathione Sulphur*	Oxidized Sulphur*
0	21	24
16	2	22
20	2	28
24	3	21

* Mg sulphur in protein-free extract/100 g of liver.

experiments on the uptake of cysteine were of short duration, generally 2 hr or less, and hence complete before the onset of this mobilization. No changes were observed in the oxidized sulphur fraction of the liver.

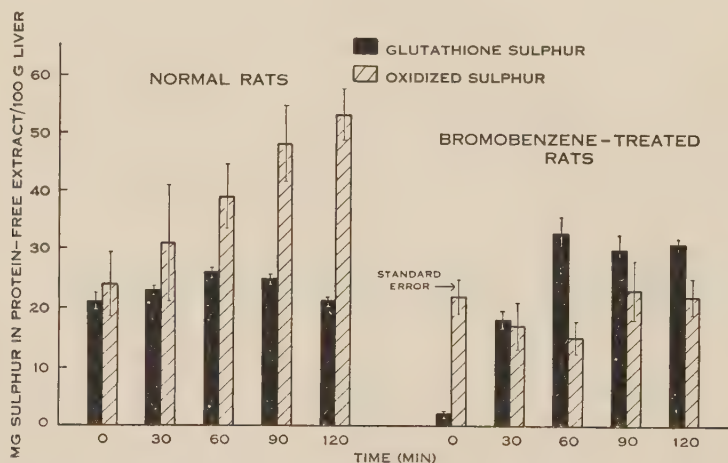


Fig. 1.—Changes in the sulphur-containing compounds of the liver after oral administration of cysteine (30 mg).

Although it has been claimed that bromobenzene depletes only the cysteine stores of the liver, it was considered advisable to check some other components. After the injection of bromobenzene no changes were observed either in the total α -amino nitrogen or in the protein concentration of the liver.

(b) The Uptake of Cysteine by the Liver

Initially, samples of liver were removed from an anaesthetized animal before and at various time intervals after dosing with cysteine. Cysteine uptake and glutathione synthesis were observed in such animals. However, this technique was tedious and it was decided to use a large number of animals and take only a single liver sample from each.

TABLE 2

DETERMINATION OF THE NATURE OF SULPHYDRYL-CONTAINING COMPOUNDS IN THE LIVER

Total sulphydryl concentration was estimated by the nitroprusside method, and the glutathione by the glutathione reductase method. Cysteine hydrochloride (30 mg) and glucose (150 mg) were dissolved in 1 ml of water and given by stomach tube at time 0

Time (min)	Normal Rats		Rats Deficient in Glutathione	
	Total Sulphydryl Sulphur*	Glutathione Sulphur*	Total Sulphydryl Sulphur*	Glutathione Sulphur*
0	18.1	18.4	3.3	3.1
60	25.2	24.7	26.8	26.8
120	22.7	22.9	28.8	28.3

* Mg sulphur in protein-free extract/100 g of liver.

Figure 1 shows that in normal livers the concentration of glutathione and oxidized sulphur was 21 and 24 mg sulphur/100 g of tissue respectively. The concentration of cysteine was too small to be estimated (<1 mg sulphur/100 g of liver). No accumulation of cysteine in the liver could be demonstrated after dosing the animals with 30 mg of cysteine. While there was only a slight increase in the concentration of glutathione, the concentration of oxidized sulphur was more than doubled. In a few animals investigated the concentration of oxidized sulphur was still above normal at 8 hr after dosing but had returned to the base line by 24 hr.

When cysteine was given to those animals in which the concentration of glutathione in the liver had been lowered beforehand, a marked and rapid increase in the concentration of sulphydryl groups occurred. This increase was not due to cysteine but could be accounted for by an increase in glutathione (Table 2). No increase, on the other hand, was observed in the oxidized sulphur fraction (Fig. 1).

(c) The Accumulation of Cysteine in the Liver

In order to obtain an increase in the concentration of cysteine it was necessary to raise the dose to 150 mg and to use the intraperitoneal route. Figure 2 shows the results obtained when cysteine was injected into rats deficient in glutathione. An increase in the concentration of glutathione occurred as before. The cysteine

concentration increased at first from <1 to 16 mg of cysteine sulphur/100 g of liver, but this increase was only temporary and had decreased to 8 mg by 60 min and was down still further (4 mg) after 120 min. With these high doses, cysteine also accumulated in plasma and in red blood cells (Eden, unpublished data). A concomitant increase of the oxidized sulphur fraction was also obtained in the tissues (Fig. 2). The same degree of cysteine accumulation was observed when normal animals were injected with these high doses.

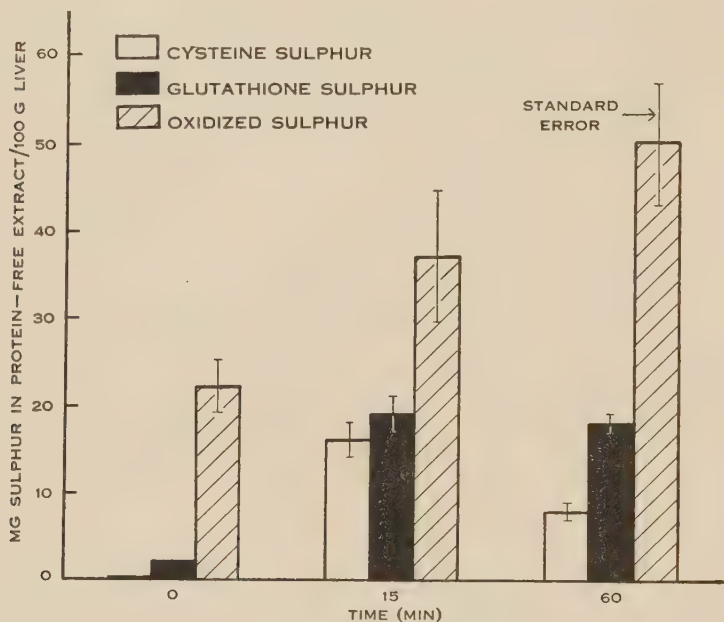


Fig. 2.—Changes in the sulphur-containing compounds of the liver after intraperitoneal injection of cysteine (150 mg).

IV. DISCUSSION

No accumulation of free cysteine occurred in the liver when 30 mg of cysteine was given by stomach tube. This amount is more than the daily intake of cysteine even on a high protein diet. Only when very high doses (150 mg) were injected intraperitoneally did cysteine accumulate temporarily. The presence of cysteine in the liver has also been observed by Awapara (1953). In these experiments likewise large amounts of cysteine were injected intraperitoneally.

When 30 mg cysteine was given by stomach tube, more than half of the sulphur was recovered in the liver of all animals dosed. In those animals in which the concentration of glutathione in the liver had first been lowered to 10 per cent. of the normal level, all the cysteine taken up was converted to glutathione. The concentration of glutathione was restored in less than 1 hr, hence the rate of synthesis was higher than in normal animals where the turnover has been estimated to be about 3 hr (Waelsch and Rittenberg 1942).

In normal animals, nearly all the cysteine taken up was oxidized. In these livers no cystine could be found although it is known that isolated liver mitochondria can oxidize cysteine to cystine (Lang and Keller 1955). Thus the increase in the concentration of oxidized sulphur was caused by the accumulation of other oxidation products of cysteine. The oxidation of cysteine to taurine and to inorganic sulphate involves a large number of intermediary products, but only taurine normally accumulates in the liver (Awapara 1953) and is eventually excreted in the bile (Portman and Mann 1955) and in the urine (Awapara 1956). When the intake of sulphur amino acids is high as with a high protein diet, more taurine (Evered 1956) and more sulphate (Hawk, Oser, and Summerson 1954) are excreted than when the intake is low. As cysteine is not excreted to any considerable degree in the urine, the oxidation of cysteine in the liver seems to be the mechanism by which cysteine not required for anabolic reactions by the body is removed. This may be compared to the role of the liver in converting the nitrogen of the amino acids to urea.

The results obtained in the present investigation suggest that when cysteine is taken up, it is rapidly converted to glutathione, and once the glutathione level is saturated the excess cysteine is oxidized. Only when both the synthesizing and the oxidizing systems are overloaded, by giving quite unphysiological amounts of cysteine, can free cysteine be detected in the liver.

In contrast to cysteine, glutathione makes up a considerable proportion (over 10 per cent.) of the amino acids present in the protein-free extracts of the liver (Ferrari and Harkness 1954). Several steps in the oxidation of cysteine to inorganic sulphate in the liver are not reversible to any significant extent (Singer and Kearney 1955); on the other hand, glutathione, if oxidized, is readily reduced by glutathione reductase present in the liver (Rall and Lehninger 1952). The rapid conversion of cysteine to glutathione may prevent the irreversible oxidation of cysteine in the cell and at the same time provide a readily available source of cysteine for protein synthesis.

V. ACKNOWLEDGMENTS

This investigation was supported by the National Health and Medical Research Council and the University of Sydney Research Fund. One of us (W.G.J.) is indebted to the National Health and Medical Research Council for a full-time grant.

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TERMINAL AMINO GROUPS IN WOOL AND *S*-CARBOXYMETHYL KERATEINE 2

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[*Manuscript received January 9, 1957*]

Summary

The *N*-terminal residues of Merino 64's quality wool and of a purified protein derivative extracted from it, *S*-carboxymethyl kerateine 2, have been determined. A similar total *N*-terminal residue content is present in both wool and *S*-carboxymethyl kerateine 2 comprising glycine, serine, threonine, aspartic acid, glutamic acid, and alanine. These occur in different proportions in the two materials and valine is an additional *N*-terminal amino acid present only in wool.

It is concluded from the quantitative data that these *N*-terminal residues are not stoichiometrically significant in either case and that cyclic structures are present.

I. INTRODUCTION

By treating wool with alkaline thioglycollate solutions a soluble protein representing at least 20 per cent. of the wool has been isolated, which behaves as a relatively pure substance during electrophoresis (Gillespie and Lennox 1955*a*, 1955*b*). This protein has been converted to the *S*-carboxymethyl derivative (Gillespie 1956) and further fractionated by precipitation with zinc ions or ammonium sulphate (Gillespie 1957).

The 1,2,4-fluorodinitrobenzene (FDNB) (Sanger 1945) and phenylisothiocyanate methods (Edman 1950) of *N*-terminal group analysis have been applied to *S*-carboxymethyl kerateine 2 (SCMK2) to investigate whether any of the known *N*-terminal residues of wool (Middlebrook 1951) have been lost during extraction of the soluble protein, or alternatively if any new *N*-terminal residues indicative of random hydrolysis have appeared, and also to compare quantitatively the *N*-terminal residues with those present in wool. It was also hoped that end-group studies would be of some value in following the course of fractionation of SCMK2.

In contrast to soluble proteins, which react quantitatively with FDNB in aqueous solution during 2 hr at 40°C, wool requires at least 48 hr for adequate penetration of the reagent (Middlebrook 1951). In the present studies on wool the method of Fritze and Zahn (1956) for the exhaustive dinitrophenylation of wool has been used to ensure maximum reaction with the *N*-terminal residues.

II. MATERIALS AND METHODS

Virgin Merino wool, 64's quality, from one fleece, was supplied by Dr. F. G. Lennox after light scouring in the following manner: the tips were removed and the wool was washed three times successively with ether and six times with water; after squeezing out excess water the wool was dried in a forced air draught at 40°C; the wool was then treated once more with ether and four times with water before

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drying for 2 hr at 40°C; finally, the wool was hand-combed and conditioned at 20°C and 63 per cent. relative humidity.

A further sample of Merino 64's wool in the form of dry combed top was also studied. This was extracted four times with light petroleum, four times with ethanol, and repeatedly with distilled water before being dried and conditioned. Samples of SCMK2 were prepared by Mr. J. M. Gillespie according to the published procedures (Gillespie 1956, 1957).

III. EXPERIMENTAL

(a) *Dinitrophenylation*

The standard conditions of Fritze and Zahn (1956) were used to dinitrophenylate wool: 1.66 per cent. sodium bicarbonate solution, 50 per cent. FDNB on the weight of wool (liquor : wool ratio of 60 : 1) were incubated at 60°C for 7 hr with changes of the reaction medium after 2½ and 5 hr. Washing of the dinitrophenylated wool was carried out according to the directions of these workers and after drying in warm air the DNP-wool was equilibrated at 63 per cent. relative humidity and 20°C.

Similar conditions were used for the dinitrophenylation of SCMK2. To 100 ml of a 1 per cent. solution was added 1.5 g NaHCO₃ and, after bringing to 60°C, FDNB (0.35 ml) was added and the mixture vigorously stirred for 2½ hr. Further amounts of FDNB were added after 2½ and 5 hr. After 7 hr the solution was extracted three times with ether in a centrifuge tube, acidified, and the suspension again extracted with ether. The precipitated DNP-protein was separated by centrifugation and washed three times with water, acetone, and ether before conditioning at 63 per cent. relative humidity and 20°C.

Since these conditions are more severe than those used with other proteins, control experiments were carried out on insulin. DNP-glycine (1.07 moles) and DNP-phenylalanine (0.91 mole) were the only DNP-amino acids detectable in hydrolysates of the DNP-insulin (Sanger 1945) showing that with this protein, at least, no splitting of peptide bonds had taken place during the dinitrophenylation.

The percentage of protein in the DNP-protein was calculated from the weight increase in the case of wool and from the amino acid composition (Simmonds 1955), assuming one DNP group was attached to each imidazole, phenolic, and amino group in the case of SCMK2. In both cases this was equal to 91 per cent. Calculation of the percentage of wool in DNP-wool from the analytical data of Simmonds (1954) also gives 91 per cent.

(b) *Hydrolysis of DNP-proteins and Extraction of DNP-amino Acids*

About 0.5 g of DNP-wool or 0.1–0.3 g of DNP-SCMK2 was hydrolysed in a sealed tube at 105°C for 16 hr with 6N hydrochloric acid (10 ml) which had been distilled three times in glass.

The hydrolysates were filtered free of humin and diluted to 50 ml. The DNP-derivatives were extracted four times with 50 ml portions of peroxide-free ether. The combined extracts were washed three times with 10 ml N HCl.

(c) *Chromatography*

Chromatography on buffered "Celite 545" (Perrone 1951) was used to isolate the DNP-amino acids from hydrolysates of DNP-wool with the techniques previously described (Thompson 1954). All the DNP-amino acids derived from wool could be separated on two columns. However, the characteristics of the "Celite 545" were somewhat different from those of the previous batch and the following method of separation was employed.

The extracted DNP-derivatives, after sublimation (Mills 1952) at 60°C to remove dinitrophenol, were taken up in water-saturated chloroform and transferred to a chloroform column of pH 3.7 (stationary phase 1 ml 2M Na₂HPO₄). The fast band was collected and when it had run off the column (2 g) the solvent was changed to a solution of 5 per cent. ether in purified chloroform which was saturated with water (CE5), when the DNP-threonine moved ahead of the DNP-glutamic acid, DNP-serine, and DNP-aspartic acid. Once the DNP-threonine was separated from the slower band the solvent was changed to CE10 which gave four distinct bands in the column with the following *R* values:

DNP-threonine	0.9-1.0	DNP-serine	0.2-0.25
DNP-glutamic acid	0.5-0.7	DNP-aspartic acid	0.1-0.15

These were collected separately for spectrophotometric estimation in 1 per cent. NaHCO₃ (Sanger 1949). The fast band from the pH 3.7 column was taken to dryness, dissolved in a small quantity of water-saturated chloroform, and transferred to a pH 6.5 chloroform column. The dinitroaniline and dinitrophenol (*R* = 1) moved rapidly through the column and DNP-valine (*R* = 0.2) was the fastest of the DNP-amino acids. Development with chloroform was continued until the DNP-valine was near the bottom of the column since a faint yellow artefact band preceded it. Then the proportion of ether in the chloroform was increased through CE10, CE25, and CE50 to water-saturated ether to separate the DNP-alanine from DNP-glycine. The DNP-glycine could be finally eluted from the column by adding ethyl acetate to the ether. The DNP-amino acids from hydrolysates of DNP-SCMK2 were estimated after two-dimensional paper chromatography using *tert*.-pentanol-pH 6 phthalate buffer as solvent in the first dimension on Whatman No. 1 paper which had been sprayed with 0.05M phthalate buffer of pH 6 (Blackburn and Lowther 1951) and 1.5M phosphate buffer (pH 6) as solvent (Levy 1954) for the second dimension. For quantitative estimation the spots were cut out and extracted with 1 per cent NaHCO₃ (Levy 1954). The aqueous phase after extraction of the ether-soluble DNP-amino acids was examined for DNP-arginine by paper chromatography in the *tert*.-pentanol-pH 6 phthalate system but none was detected in either DNP-wool or DNP-SCMK2.

(d) *Reaction with Phenylisothiocyanate*

The procedure used for the coupling was that of Fraenkel-Conrat and Singer (1954) using reagents purified as described by Fraenkel-Conrat, Harris, and Levy (1955). To 5 ml of 1.5 per cent. SCMK2 solution in a 30-ml beaker were added 5 ml of purified dioxan. The solution was brought to 40°C, stirred magnetically, and

the pH was adjusted to 8.5 with 0.1N KOH using an external glass-electrode assembly. Phenylisothiocyanate (0.1 ml) was added and the mixture was stirred at 40°C for 1½ hr maintaining the pH constant by addition of 0.1N KOH. The solution was brought to pH 9, transferred to a centrifuge tube made from a "Quickfit" B24 socket of 40-ml capacity with several washes of water, and extracted with equal volumes of cyclohexane (2×) and benzene (5×). The aqueous solution was then freeze-dried in the same tube. Preliminary experiments on the cyclization of phenylthiocarbamyl (PTC)-insulin, prepared in a similar way, at temperatures of 100, 70, and 40°C using 1N and 4N HCl for periods of time ranging from ½ to 24 hr showed that the maximum yield of phenylthiohydantoin (PTH) was obtained using 4N HCl at 70°C for 30 min or 40°C for 18 hr. At 100°C 2N HCl gave better yields of PTH after 30 min than did 1N or 4N HCl. With longer heating at 100°C yields decreased in all cases but most sharply at the 4N concentration.

For cyclization of PTC-SCMK2, heating in 4N HCl at 70°C for 30 min was therefore employed. The residue was dissolved in 1.33 ml water and 2.67 ml redistilled 6N HCl added to make a final concentration of 4N HCl. The mixture was heated in the B24 tube fitted with a condenser for 30 min at 70°C with periodic shaking. The solution was then cooled, diluted with 4 ml water, and extracted with 8 ml ethyl acetate (3×). The combined ethyl acetate extracts were washed with 2 ml water (2×) and made up to 25 ml. Aliquots were taken, the ethyl acetate removed *in vacuo*, and the residue dissolved in optically-pure ethanol for spectrophotometric measurements in the range 240–290 mμ. The solutions were then concentrated to dryness and hydrolysed with 6N HCl at 150°C for 18 hr to regenerate the free amino acids.

IV. RESULTS

The results of replicate determinations of the *N*-terminal amino acids in two samples of the 64's virgin wool dinitrophenylated several months apart are shown in Table 1. In Table 2 the results obtained on 64's virgin wool and 64's top together with the results on samples of SCMK2 are presented. The values for 64's top are from a single determination and for the SCMK2 samples the results are the mean of duplicate determinations.

In view of the correction factors which have been applied to allow for the breakdown of DNP-amino acids during hydrolysis of DNP-proteins the values in Table 2 do not have a high order of accuracy. The correction factors used have been based on the recoveries of the DNP-amino acids reported by Porter and Sanger (1948) and Middlebrook (1951). No attempt has been made to determine them under the conditions of the experiments in view of the difficulty of getting an adequate control, as emphasized by the recent difficulties reported by Geschwind and Li (1956) with α -corticotropin. With SCMK2 the results obtained with the phenylisothiocyanate procedure suggest that these correction factors are of the correct order.

Table 2 shows the values obtained for the *N*-terminal residues of several SCMK2 preparations. Fractionation of the SCMK2 preparations by Zn⁺⁺ precipitation has reduced the number of *N*-terminal residues to a level similar to that

present in wool. In unfractionated SCMK2 small quantities of *N*-terminal leucine were detected which is not present as an *N*-terminal residue in wool. This could

TABLE 1
N-TERMINAL AMINO ACIDS IN VIRGIN 64'S WOOL
Values in $\mu\text{moles/g}$ DNP-protein

Terminal Amino Acid	Sample 1				Sample 2		Mean $\pm$ 95% Confidence Limits
	1	2	3	4	1	2	
Aspartic acid	0.39	0.36	0.41	0.32	0.28	0.27	0.34 $\pm$ 0.04
Glutamic acid	0.57	0.61	0.87	0.72	0.60	0.58	0.66 $\pm$ 0.13
Serine	1.32	1.28	1.22	1.14	1.19	1.23	1.23 $\pm$ 0.08
Threonine	4.54	(4.91)*	4.35	4.33	4.34	4.50	4.41 $\pm$ 0.16
Glycine	1.00	1.04	0.90	1.10	1.21	1.16	1.07 $\pm$ 0.09
Alanine	0.85	0.96	0.93	(1.38)*	0.70	0.80	0.85 $\pm$ 0.09
Valine	0.97	1.05	0.93	1.08	1.03	0.91	1.00 $\pm$ 0.08

* The values in parenthesis were omitted in the statistical analysis.

imply a small degree of peptide bond hydrolysis during the isolation of SCMK2 from wool.

TABLE 2
N-TERMINAL AMINO ACIDS IN WOOL AND S-CARBOXYMETHYL KERATEINE 2 (SCMK2)
Values in $\mu\text{moles/g}$ protein

Terminal Amino Acid	Recovery Assumed (%)	Virgin 64's $\pm$ 95% Confidence Limits	64's Top	Unfractionated SCMK2*	Fractionated SCMK2	
					Zn Ppt.†	Zn Ppt. Washed Twice‡
Aspartic acid	70	0.5 $\pm$ 0.1	0.5	} 3.6	1.2	0.8
Glutamic acid	67	1.1 $\pm$ 0.2	0.7		3.1	1.6
Serine	80	1.7 $\pm$ 0.1	1.2		3.7	5.0
Threonine	86	5.6 $\pm$ 0.2	4.1		2.6	2.2
Glycine	15	7.8 $\pm$ 0.7	8.6		8.2	5.7
Alanine	64	1.5 $\pm$ 0.2	1.3	1.1	0.7	0.9
Valine	64	1.7 $\pm$ 0.1	1.6	0.6	0	0
Leucine	64	0	0	0.7	0	0
Total		19.9	18.0	22.7	19.5	16.2

* Preparation No. Z2F.

† Preparation No. Z4F.

‡ Preparation No. Z8F.

Recent work by Harrap (unpublished data) has shown that kerateine 2 may be extracted from wool during 18 hr at pH 10.5 and 4°C, conditions which are probably milder than those used by Gillespie and Lennox (1955*a*) for the isolation of the preparations used in the present work.

Figure 1 shows the absorption curves of the PTH derived from a preparation of PTC-insulin and PTC-SCMK2. The PTC-SCMK2 preparation showed only small absorption in the region 265–270 $m\mu$ where PTH show their maximum. The concentration of the PTH in the ethanol solution was calculated from the absorption at 267.5 $m\mu$ using a value of 16,000 as the average molar extinction coefficient (Fraenkel-Conrat, Harris, and Levy 1955). This gave a value of 19.9 μ moles/g SCMK2 for the number of *N*-terminal residues, in good agreement with the figures obtained by the DNP-technique. The amino acids formed by hydrolysis of the PTH were identified by paper chromatography with 75 per cent. (w/w) phenol–water in an atmosphere

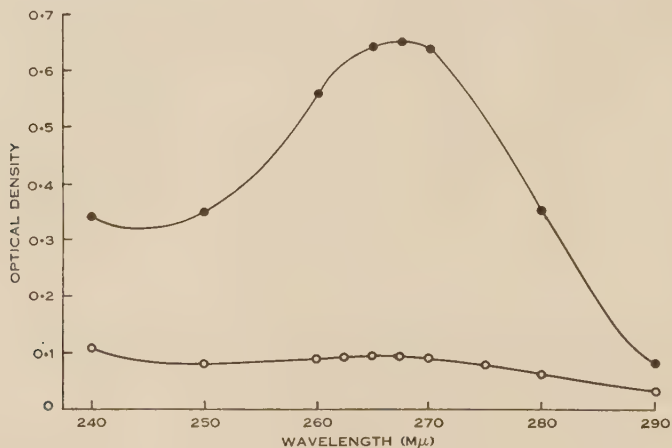


Fig. 1.—Absorption curves of the phenylthiohydantoins derived from phenylthiocarbamyl-insulin (●) and phenylthiocarbamyl-SCMK2 (○). The optical densities are for the extract from 0.25 μ mole of insulin and 3 mg of SCMK2 in 10 ml of ethanol.

of NH_3 and HCN. Only small amounts of glutamic acid and glycine together with traces of aspartic acid, alanine, and α -aminobutyric acid were detected. Dr. J. H. Bradbury (unpublished data) has studied this SCMK2 preparation and wool for *C*-terminal residues by the hydrazinolysis technique. A similar low content of *C*-terminal residues was found for both wool and SCMK2 of the same order as for the *N*-terminal residues.

V. DISCUSSION

Qualitatively the results on wool agree with those of previous workers (Blackburn 1950; Middlebrook 1951; Alexander and Smith 1956). The quantitative estimates of the total *N*-terminal residues of the samples of wool used in this study (18–20 μ moles/g protein) are in reasonable agreement with those of Middlebrook (1951) who used Lincoln fleece wool (16.8 μ moles/g) and for New Zealand Romney wool and Australian Merino wool (16.2 μ moles/g) quoted by Speakman (1955). In view of the known heterogeneity and variability of wool fibres, and the difficulty of obtaining a suitable correction factor for the losses of DNP-amino acids that occur during peptide bond hydrolysis and the different techniques used for the preparation

and hydrolysis of the DNP-proteins, differences such as these are not surprising. However, the results of Alexander and Smith (1956) using a Botany yarn are considerably lower ($11.6 \mu\text{moles/g}$).

Attempts to reduce the number of *N*-terminal residues in wool by electro dialysis in water (3 days) or in 0.2N acetic acid (4 days) or by Soxhlet extraction with water at 90°C for 24 hr did not significantly alter the values on virgin 64's wool. Moreover, when dinitrophenylation was carried out in 6M guanidine hydrochloride solution at 60°C for 7 hr or at pH 13 and 40°C for 3 hr the *N*-terminal residues were not significantly increased.

Compared with the arginine ($602 \mu\text{moles/g}$) and lysine ($193 \mu\text{moles/g}$) residues (Simmonds 1954) the *N*-terminal residues represent only a minor proportion of the basic groups in wool.

It is doubtful whether the observed *N*-terminal residues in wool can be interpreted in terms of a molecular model along the lines suggested by Middlebrook (1951). The lack of a well-defined molecule makes interpretation difficult but it would seem more profitable to regard the *N*-terminal residues as representing impurities or breakage points in a collection of cyclic molecules held together by disulphide linkages rather than representing discrete polypeptide chains. Woodin (1956) has recently examined feather keratin in some detail and has proposed cyclic structures for this keratin molecule which appears to have a definite molecular weight. The *N*-terminal residues in feather keratin are qualitatively similar to those in wool keratin and were attributed to impurities or hydrolysis of peptide bonds during the coupling reaction.

If wool protein is composed of a series of different polypeptide chains held together by disulphide linkages, then it might reasonably be expected that methods of fractionation applied subsequently to disulphide bond rupture would give fractions substantially free of some of the end-groups of wool. In fact, however, oxidation of the disulphide linkages followed by fractionation into α , β , and γ keratoses, representing 60, 10, and 30 per cent. of the wool respectively, has given proteins with exactly the same *N*-terminal groups as the original wool although the proportions do vary (Alexander and Smith 1956).

Reduction followed by fractionation has produced similar results as outlined in this paper. SCMK2 represents only about 20 per cent. of the wool yet contains the same *N*-terminal residues with the exception of valine. In view of the fact that no pure protein has given more than three different *N*-terminal residues, as in the case of α -chymotrypsin (Bettelheim 1955), while most have one or two terminal residues (Sanger 1955), it seems probable that the *N*-terminal residues observed in SCMK2 and wool are not stoichiometrically significant and represent slightly degraded cyclic molecules. For SCMK2, estimates of the molecular weight have been made in this Laboratory. Harrap (1956) found a value of about 8000 for the molecule unfolded at the surface of a solution of low ionic strength, whereas, when dissolved in a solution of high ionic strength at pH 7, O'Donnell and Woods (1956) found aggregation occurred to give values of the order of 200,000. In urea or sodium dodecyl sulphate solution disaggregation to particle sizes of 45,000-50,000 occurred and lower values of 20,000-30,000 were estimated for solutions of pH 13. For

none of these observed molecular weights would the observed *N*-terminal residues be stoichiometrically significant.

It is of some interest that collagen, which has no detectable *N*-terminal residues (Bowes and Moss 1953; Grassman and Hörmann 1953), on conversion to gelatin gives rise to glycine, serine, threonine, aspartic acid, glutamic acid, and alanine as the principal *N*-terminal residues (Courts 1954) regardless of whether acid- or alkali-processing is used and irrespective of the origin of the collagenous precursor. These are the same *N*-terminal residues as are detectable in wool and wool proteins.

VI. ACKNOWLEDGMENT

The author wishes to thank Mr. W. B. Hall for the statistical analysis.

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THE STRUCTURE OF INEFFECTIVE ROOT NODULES OF LEGUMES: AN UNUSUAL NEW TYPE OF INEFFECTIVENESS, AND AN APPRAISAL OF PRESENT KNOWLEDGE

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[Manuscript received March 6, 1957]

Summary

A new type of symbiotic ineffectiveness in subterranean clover is described in which very slow-growing nodules are produced. In these the nodule bacteria are not released from the infection threads, where they multiply at the expense of the host tissue. Comparison of nuclear size of normal effective nodules with that of these ineffective nodules suggests that the nodule tissue is almost devoid of the disomatic cells in which the nitrogen-fixing bacteroid form of *Rhizobium* normally develops. The bacterial strains giving rise to these nodules were obtained as stable variants of a strain producing structurally and cytologically normal nodules.

Other forms of structural abnormalities, resulting in symbiotic ineffectiveness in legume root nodules, are discussed and it is concluded that the bacteria must be fully developed to a non-viable but metabolically active bacteroid form within special host cells before nitrogen fixation can take place.

I. INTRODUCTION

Variations in nitrogen-fixing capacity and infectivity of strains of *Rhizobium* have often been reported. For example, Almon and Baldwin (1933) and Nutman (1946) found a range of variants of nodule bacteria under various culture and storage conditions. Other workers have found non-infective variants of nodule bacteria in culture and after growth in commercial peat culture. In some of these, serological characters remained unchanged (Brockwell 1954; Vincent 1954; Waters 1954; Marshall, unpublished data.)

A strain of *Rh. trifolii* (SU220), of intermediate effectiveness on subterranean clover (*Trifolium subterraneum* L.) has been found to be unstable both in culture and during growth in the host plant rhizosphere. Nutman (unpublished data) found that variant colonies continually appeared in culture and most of these were non-infective. In addition, two further substrains (220,1 and 220,2), which produced slow-growing cortical swellings on the roots of subterranean clover, were isolated from the rhizosphere of plants inoculated with the stock strain. The cortical swellings ultimately assumed the external appearance of small nodules, but the host plants showed no evidence of nitrogen fixation, dry weights of 100-day-old plants being the same as those of uninoculated control plants.

This report describes the structure of these ineffective nodules and the implications of the findings are discussed. In addition, an appraisal is made of the known structural abnormalities which result in symbiotic ineffectiveness. These abnormalities are discussed in relation to the development of the structures essential for symbiotic nitrogen fixation in legume root nodules.

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II. MATERIALS AND METHODS

Subterranean clover, Mt. Barker variety, was used throughout as host and plants were grown from surface-sterilized seed in 7/8 by 6 in. test tubes on sterile, nitrogen-free, seedling-nutrient agar (Thornton 1930a). The seedlings were inoculated immediately after germination and then grown under good growth conditions in a glass-house.

Nodules of various ages were cut from the roots and fixed overnight in Flemming's solution, after which they were dehydrated through a graded alcohol series, embedded in paraffin wax, and cut into serial microtome sections 3–5 μ thick. These were stained by the rose bengal-light green method (Brenchley and Thornton 1925), and by Heidenhain's haematoxylin. The structure of the ineffective nodules produced by the bacterial substrains 220,1 and 220,2 was compared with that of normal, intermediately effective nodules produced by the parent strain, SU220, and that of fully effective nodules produced by strains SU297 and SU298.

III. RESULTS

The structure of the young nodules showed that these cortical swellings did in fact contain the main host structures characteristic of nodules. Pairs of vascular strands with their endodermi were found within a fairly thick cortical layer. Meristematic tissue was present at the distal extremities of the nodules, but only a few mitotic figures were seen in all the material examined. This was not surprising in view of the observed slow growth of these structures. The centres of the nodules were devoid of free bacteria although the net of infection threads was present (Plate 1, Fig. 1); these showed the usual tendency to grow towards the host nuclei and developed vesicles of various sizes but none of them was ruptured. The host cells containing unbroken infection threads were larger than uninfected cells but were otherwise very different from infected cells of effective nodules. They contained little cytoplasm and their nuclei were about the same size as the small nuclei of nodule cortical cells of the same age (Fig. 1). Within effective nodules produced by the other strains of bacteria, release of bacteria from the threads followed closely upon penetration of the host cells, in which the nuclei were initially at least twice the dimensions of those of cortical cells of the same age, and then rapidly became hypertrophied as bacteria multiplied in the cytoplasm and developed into bacteroids, (Fig. 1(d)).

Older nodules (Plate 2) showed a great deal of degeneration in the oldest, proximal tissue, but the main feature was the persistence of the unbroken infection threads through the remaining intact tissue. These threads were very much enlarged and were filled with masses of rod-shaped bacteria (Plate 1, Fig. 2). The host cells through which these giant threads passed were devoid of nuclei and were degenerate. In the completely degenerate regions at the bases of older nodules the threads were ruptured, and bacteria were free in the remains of the host tissue. In three of these nodules several host cells containing bacteroids, or their remains, were seen in the oldest parts of the central tissue. These cells closely resembled the cells of the central tissue of effective nodules.

From the above observations it will be seen that the ineffective nodules produced by the substrains 220,1 and 220,2 were characterized by the failure of the bacteria to escape from the infection threads; the central tissue then degenerated as the bacteria multiplied within the threads.

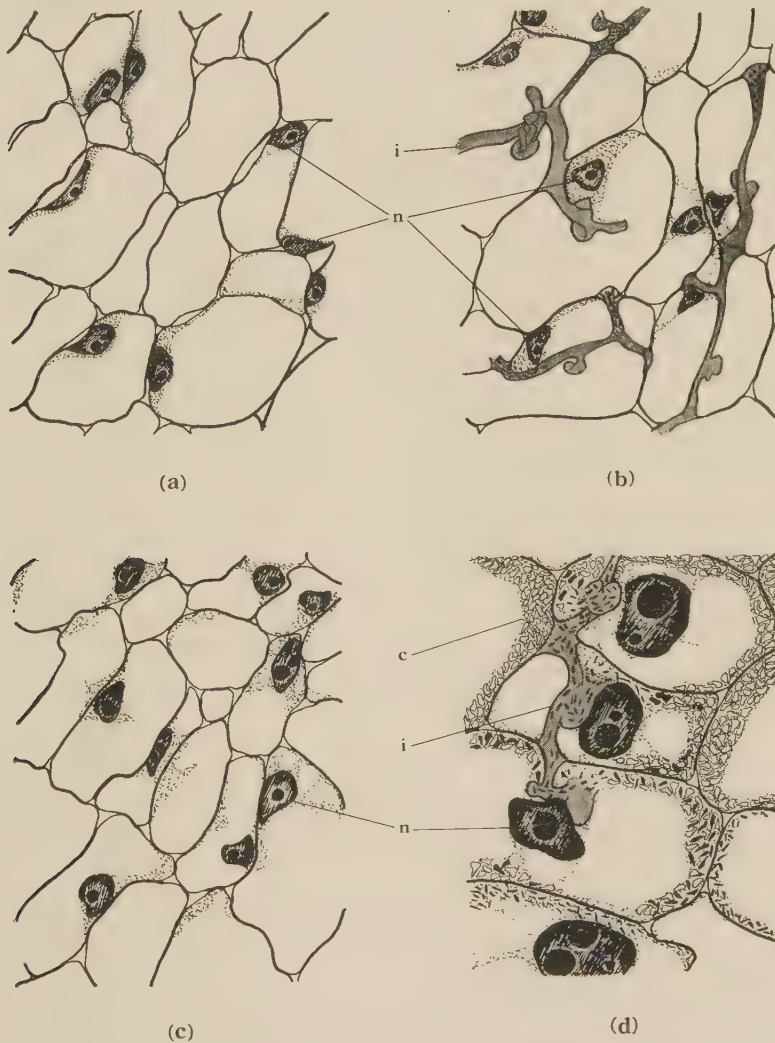


Fig. 1.—Comparison of mono- and disomatic nodule tissue. Camera lucida drawings. (a) Diploid cortical cells showing small nuclei—220,1 nodule; (b) central tissue of the same 220,1 nodule showing small nuclei and unbroken infection threads; (c) cortical tissue of an effective subterranean clover nodule; (d) young bacteroid-containing tissue of the same effective nodule. *i*, Infection threads; *n*, nuclei; *c*, cytoplasm containing bacteroids.

IV. DISCUSSION

In the type of ineffective nodulation described above there was a failure of the establishment of a symbiosis in which modified, metabolically active but non-viable bacteroid forms of *Rhizobium* (Almon 1933) are found in close association with

host cytoplasm in specialized nodule tissue (see reviews of nodule structures by Allen and Allen 1954 and Nutman 1956). Instead the bacteria were associated with degeneration of the host tissue while still within infection threads which remained intact until the tissue had completely broken down. Similar enlarged infection threads have been described in nodules of plants grown in the dark (Thornton 1930b).

In seeking an explanation of this type of "symbiosis" three features of these nodules should be borne in mind:

- (i) The non-rupture of the infection threads within the cells of the central nodule tissue (although small vesicles usually developed).
- (ii) The nuclei of the cells of the central tissue were small and they rapidly degenerated after penetration of the cells by the threads, whereas those of effective nodules were initially larger and became hypertrophied after penetration of the cells by the threads.
- (iii) The oldest parts of some nodules contained cells with bacteroids.

Wipf and Cooper (1938, 1940), Wipf (1939), and Fujita and Mitsuishi (1953) have shown, in a wide range of legumes, that the central tissue of effective nodules develops from the occasional disomatic cells (with nuclei containing twice the somatic number of chromosomes) in the cortex of legume roots. These disomatic cells and some of the monosomatic cells nearby are stimulated to division by the proximity of infection threads growing from the site of initial infection, and constitute the incipient nodule meristem. This in turn produces the nodule with diploid (or monosomatic) cortex, vascular system, endodermi, etc. and a central disomatic tissue, into which alone bacteria are released from the infection threads. The nuclei of the cells of the latter tissue are readily distinguishable by their larger size relative to the dimensions of the cells, and they rapidly become hypertrophied as bacteria multiply and form bacteroids in the cytoplasm. By contrast, cortical cells traversed by infection threads generally enlarge only slightly, the contents becoming granular and the threads growing towards the nuclei which begin to degenerate. No release of bacteria occurs in monosomatic cells (McCoy 1932; Schaede 1941; Allen and Allen 1954; Nutman 1956).

It is clear that the central tissue of the nodules under consideration resembles the cortical monosomatic tissue rather than the disomatic central tissue of effective nodules. In an attempt to confirm this resemblance, 22 nodules produced by substrain 220,2 were examined for mitoses. Some nodules had no mitoses at all but 15 had from one to five when examined in serial sections. Of these the chromosomes of 16 could be counted and all were diploid ($2n = 16$).

The cortex-like nature of the central tissue suggests that the invading bacteria failed to stimulate the continued growth of the disomatic tissue, and the nodules became mere cortical outgrowths in which a few of the disomatic cells of the primordia sometimes persisted as the bacteroid-filled cells noted in (iii) above: these last presumably becoming infected and subsequently developing in the manner characteristic of effective nodules.

V. STRUCTURAL BARRIERS TO SYMBIOTIC NITROGEN FIXATION IN LEGUME ROOT NODULES

Interest in the structure of legume root nodules originally developed with the intense interest in these organs aroused by the work of Hellriegel (1887) and Hellriegel and Wilfarth (1888), who proved beyond all doubt the essential role of root nodules in the fixation of atmospheric nitrogen by leguminous plants. Fred, Baldwin, and McCoy (1932) summarized the facts known about nodule structure up to that date and accounts by Thornton (1930a), Bond (1948), Allen and Allen (1950, 1954), and Nutman (1956) contain further detailed references. From these and the author's own observations the formation of the various stages of nodule production have been set out in diagrammatic form in steps 1-8 in Table 1.

The purpose of this section is to outline the known abnormalities in nodule structure which prevent nitrogen fixation and to examine these abnormalities in an attempt to relate them to the general biology of nodules. For the sake of clarity, headings corresponding to the various steps of nodule formation, as set out in Table 1, are given, and the abnormalities occurring at each step are briefly described.

Step 1: Growth of Rhizobium in the Host Rhizosphere

While this is not a structural consideration, it is included because an appreciable rhizosphere population of nodule bacteria is a prerequisite for nodule development. Under most soil conditions nodule bacteria grow mainly in the presence of plant roots, where stimulation of growth by the various constituents of root exudates occurs. Rhizosphere growth of *Rhizobium* can be inhibited by soil acidity, and under such conditions nodules develop on inoculated plants only near the site of inoculation (Anderson, unpublished data). Hely, Bergersen, and Brockwell (1957) have also shown that rhizosphere populations of *Rh. trifolii* may be limited by microbiological antagonism in soil. Harris (1954) introduced the concept of "incur-siveness" into problems of colonization of the rhizosphere by nodule bacteria in competition with other microorganisms.

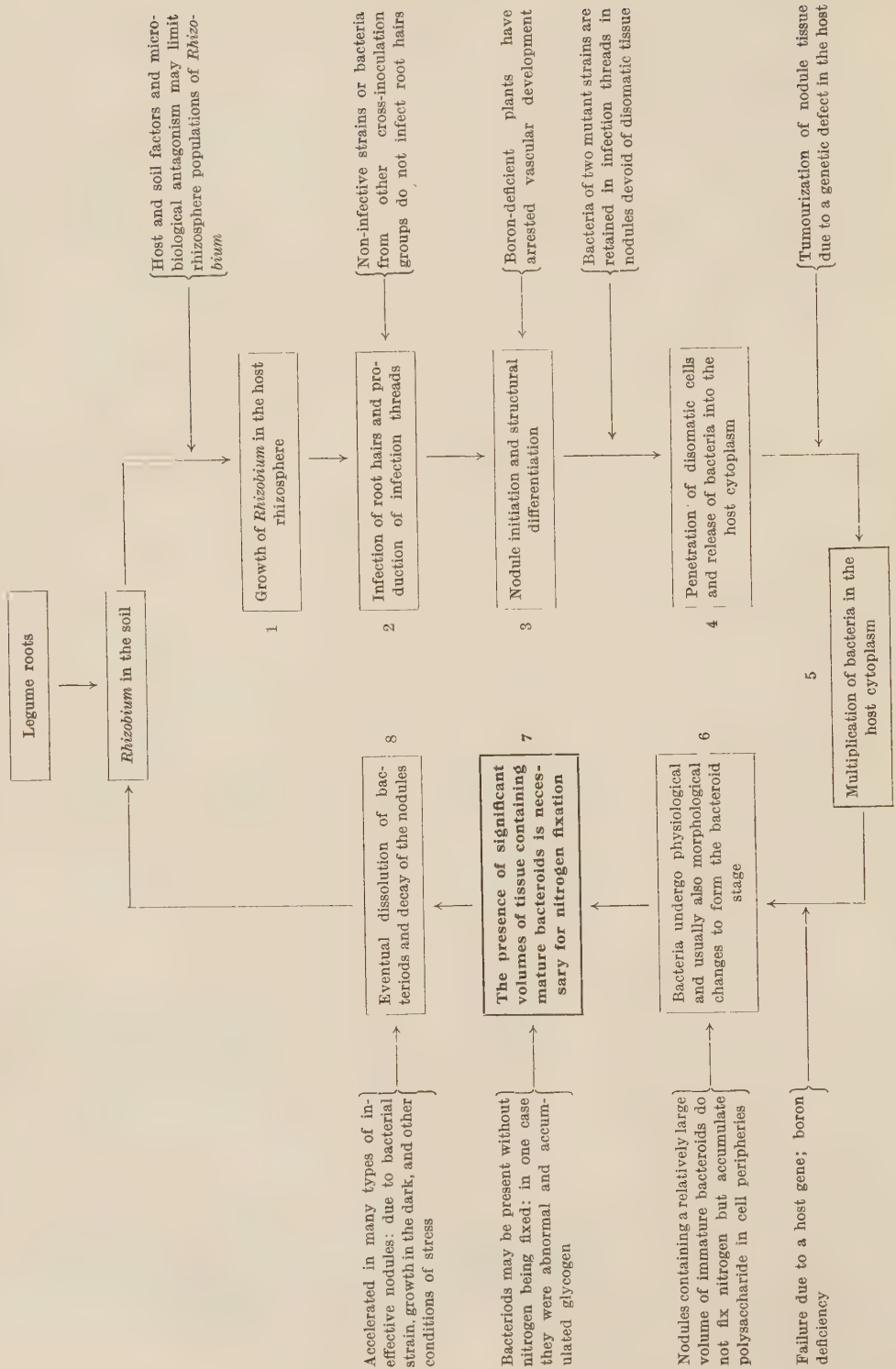
Step 2: Infection of Root Hairs and Production of Infection Threads

There is a barrier at the external surface of the host root (the root hair) which prevents bacteria capable of infecting legumes of certain genera from infecting others. This is the basis for the division of all legumes and their nodule bacteria into the so-called "cross-inoculation groups". This barrier to infection is also manifest when some mutant strains of bacteria become non-infective for the host plant, although serological and other characteristics may remain unaltered.

Step 3: Nodule Initiation and Structural Differentiation

It is at this stage that the first structural barriers to nitrogen fixation in legume root nodules are found. Brenchley and Thornton (1925) showed that vascular development was arrested in boron-deficient *Vicia faba* L. nodules. The results

TABLE 1
THE DEVELOPMENT OF ROOT NODULES IN LEGUMES AND THE KNOWN BARRIERS WHICH PREVENT NITROGEN FIXATION



described in the first part of this paper strongly suggest that bacterial mutations may prevent the stimulation of normal meristematic activity in nodules, and there is, in this case, the secondary effect of non-release of bacteria from infection threads due to the monosomatic nature of the central nodule tissue.

Step 4: Penetration of Disomatic Cells and Release of Bacteria into the Host Cytoplasm

Nutman (1957) found a recessive host gene (*ie*) in red clover, which, in homozygous plants, resulted in ineffective symbiosis with strains of bacteria normally effective on this host. Bergersen and Nutman (1957) showed that this was due to a tumourization of the central nodule tissue which followed immediately after release of bacteria from infection threads. Further nodule development was thus prevented and no nitrogen fixation could consequently take place.

Step 5: Multiplication of Bacteria in the Host Cytoplasm

This normally occurs immediately the bacteria are released from infection threads, the host cytoplasm becoming packed with bacteria in the form of uniform rods. Brenchley and Thornton (1925) observed in boron-deficient *V. faba* nodules that development of bacteria-containing tissue stopped at this point, no nitrogen being fixed. It was not clear whether this failure of development was a direct result of the deficiency in the host plant nutrition, or an outcome of the arrested vascular development mentioned in step 3.

A host gene (*i*₁) in red clover has been described (Nutman 1954*b*) which confers in the homozygous condition a fully ineffective response in plants nodulated by the usually effective strain of nodule bacteria Rothamsted A. This ineffective response was found to be due to a failure of further development of the rod-shaped bacteria in the cytoplasm of the central nodule tissue; all host structures appeared quite normal and the usual hypertrophy of the nuclei of infected cells took place (Bergersen and Nutman 1957).

Step 6: Bacteroid Formation

It is generally conceded that bacteroid formation is a prerequisite for nitrogen fixation. Bacteroids develop from the bacterial rods in the cytoplasm of disomatic host cells, which become swollen, metabolically distinct, and biologically non-viable (Almon 1933). In clover nodules, the author has described the structural changes involved in bacteroid formation; there is degeneration of the bacterial chromatin and the appearance of a somewhat transparent perinuclear area. The metabolically active mitochondria-like granules are also altered, and there is evidence that, although the constitutional enzyme systems remain (Burris and Wilson 1939) the balance between them is considerably altered (Bergersen 1955, and unpublished data).

Ineffective nodules on *Trifolium ambiguum* M. Bieb. have been found to fail in nitrogen fixation because the bacteroids do not complete the changes which are described above. Instead there is a persistence throughout the nodule of immature tissue (Bergersen 1957).

Step 7: The Persistence of Mature Bacteroids

The stage of nodule development covered by this heading seems to be the critical one for active nitrogen fixation and it is therefore emphasized in Table 1. However, bacteroids may sometimes persist in non-nitrogen-fixing nodules. In one case, such bacteroids were found to accumulate glycogen in malformed cells (Bergersen 1955). It may be possible to find, in such cases as this, biochemical barriers which intervene between processes essential for fixation.

Step 8: Eventual Dissolution of Bacteroids and Decay of the Nodules

This final stage is accelerated in some conditions, leading to reduced fixation, and in the extreme cases, to complete ineffectiveness. Most ineffectiveness has been assigned to the transitory nature of bacterial tissue in nodules produced by unsuitable strains of bacteria (e.g. Chen and Thornton 1940). Accelerated nodule decay also occurs when nodulated plants are grown in the dark or in the presence of relatively high concentrations of nitrate (Thornton 1930*b*; Thornton and Rudolf 1936). In the latter case there is also thickening of the cell walls of meristem and endodermal cells. There are probably many other conditions of stress which cause accelerated decay of bacteroid-containing tissue within nodules. For example, in plants grown for a period on nitrogen-free agar medium before inoculation, the bacteroid-containing tissue of the nodules eventually produced persists in inverse proportion to the delay in inoculation (Hely and Bergersen, unpublished data).

The development of nitrogen-fixing nodules, and the structural abnormalities which prevent fixation, all accentuate the essential role of the intracellular forms of nodule bacteria. Some workers have stated that the bacteroid form of *Rhizobium* is not indispensable to nitrogen fixation (e.g. Smith 1949, who cites as an example the rod-shaped intracellular bacteria of soybean nodules) but the facts are that unless fully developed bacteroids persist in the special disomatic host cells in many species of legumes, no fixation occurs. Bacteria, as unchanged rods, in large numbers within infection threads, or free in the host cytoplasm, do not fix nitrogen. Nor do the immature bacteroids found in some ineffective nodules on *T. ambiguum*. However, it is the author's opinion that the structural changes which occur when nodule bacteria become bacteroids are incidental to changes in metabolism which take place during the process. These changes may result from the nuclear hypertrophy which occurs in the bacteria (Bergersen 1955). In some nodules, such as those of soybeans (*Glycine max* (L.) Merrill), the intracellular form of *Rh. japonicum* is little different, in shape or size, from that of the bacteria in culture, but there are indications of relative changes in enzymatic activity, such as occur in the swollen, intracellular forms from clover nodules (Bergersen, unpublished data).

In conclusion it may be stated that for symbiotic nitrogen fixation the host must be capable of being stimulated by appropriate strains of *Rhizobium*, to produce structures composed of various tissues, both diploid and tetraploid. The bacteria must then undergo a series of changes, both structural and functional, after a period of multiplication in the cytoplasm of the tetraploid host cells, and during this process they lose their ability to divide under normal cultural conditions. The system thus established must then be capable of being either maintained for significant periods

STRUCTURE OF INEFFECTIVE ROOT NODULES OF LEGUMES

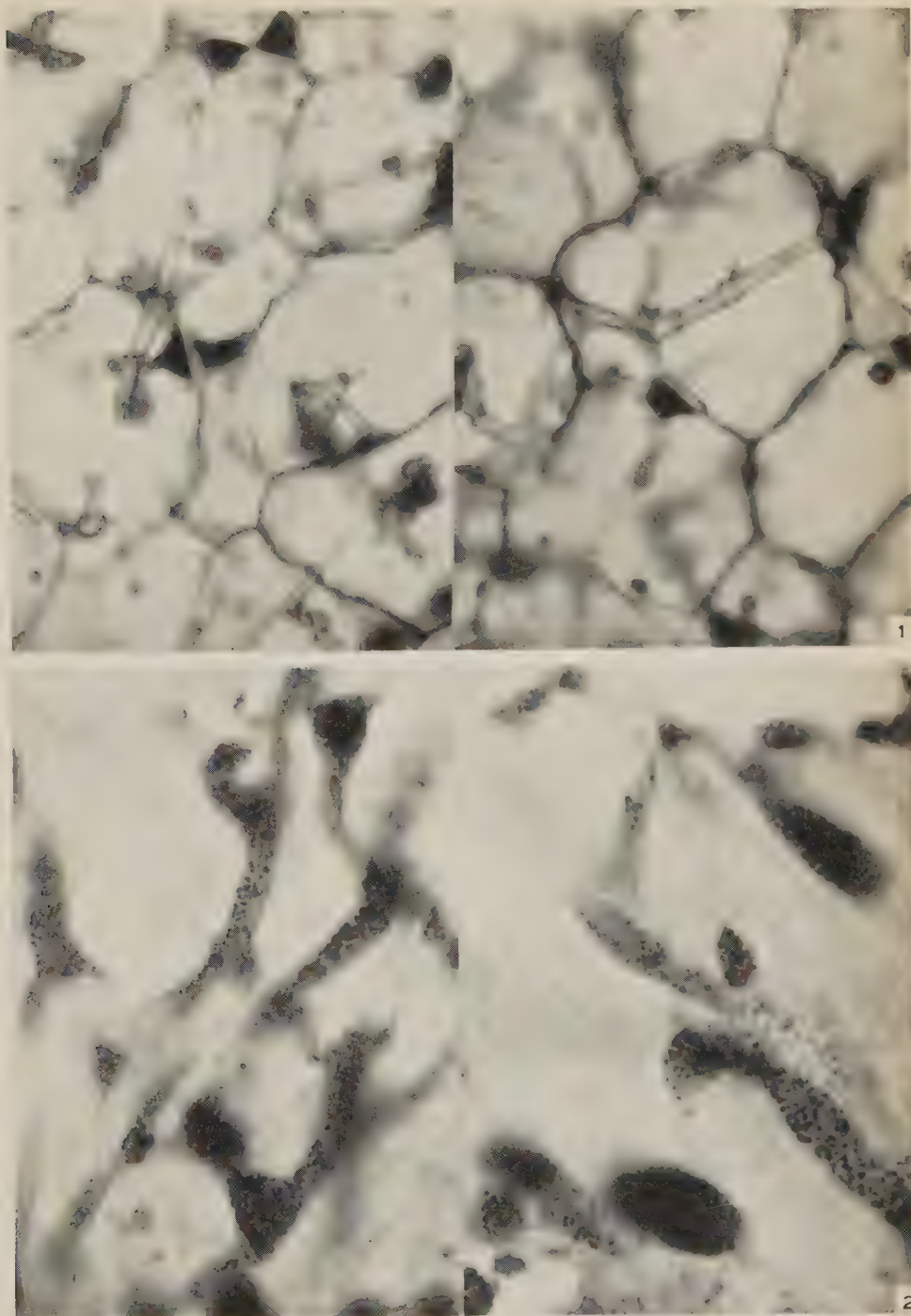


Fig. 1.—Young 220,1 nodule section showing the unbroken infection threads in cortex-like cells of the central tissue. Rose bengal-light green stain. $\times 1650$.

Fig. 2.—Enlarged infection threads within degenerating tissue of a 220,1 nodule. Rose bengal-light green stain. $\times 1650$.

STRUCTURE OF INEFFECTIVE ROOT NODULES OF LEGUMES



A mature 220,2 nodule showing degenerate host tissue and *ruminis* of bacteroid-containing cells at *b*. Rose bengal-light green stain. 200.

of time, as in most spherical nodules, or of being continuously renewed, as in most types of elongated nodules. Only when all these conditions have been fulfilled does the host plant benefit to any significant degree.

VI. ACKNOWLEDGMENTS

The author wishes to thank Dr. P. S. Nutman, Mr. A. J. Anderson, Mr. F. W. Hely, and Mr. K. C. Marshall for permission to quote unpublished results.

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THE ASEPTIC CULTURE OF *ARABIDOPSIS THALIANA* (L.) HEYNH.

By J. LANGRIDGE*

[Manuscript received January 11, 1957]

Summary

Arabidopsis thaliana (L.) Heynh. is a small, rapidly growing crucifer with a 28-day life cycle, a low chromosome number ($n=5$), and a high fertility (152 ± 11 seeds under aseptic conditions). The small size and rapid growth of the plants enable them to be grown throughout their life cycles on sterile, nutrient agar in ordinary test tubes. Alternatively, up to 50 plants may be grown on silica gel in crystallizing dishes. For optimal growth the trace element cations should be chelated and light should be excluded from the roots. When supplied with sucrose the plants absorb only the glucose portion. During growth the plants excrete small quantities of vitamins and other substances into the medium but not in amounts likely to prevent the detection of growth mutants through cross-feeding.

I. INTRODUCTION

The methods described below for the culture of *Arabidopsis thaliana* (L.) Heynh. were developed as a necessary preliminary to the study of the physiological genetics of flowering plants. For such work, the conditions of culture should meet two requirements. Firstly, the medium should be nutritionally inert except for those inorganic elements or radicals necessary for plant growth; and secondly, plants should be maintained in an aseptic condition throughout their life cycles.

The detection of mutants which occur with low frequency even after mutagenic treatment requires, especially with a diploid organism, that a relatively large number of plants be grown under closely defined conditions. However, the methods of aseptic culture that have previously been published are unsuitable for the present purposes, because in practice they allow the culture of only one or a few plants and these for usually only a part of their life cycle.

There appear to be only two published reports of a phanerogam completing its life cycle under completely sterile cultural conditions. The first of these (Laibach 1943) states that *A. thaliana* may be grown in test tubes on nutrient agar from germination to seed ripening, but no details are given. The second account (Loo 1946) concerns the culture of the desert ephemeral, *Baeria chrysostoma* T. & G. Seeds of *Baeria* were germinated on filter paper and the seedlings were transferred to an agar or sand medium in 300 by 25 mm tubes. The plants flowered after about 30 days and set some seed which, however, had low viability (less than 10 per cent. germination).

It is evident that successful aseptic culture on an adequate scale is mainly dependent on the nature of the experimental plant. Considerations of space, generation time, and genetic suitability require a plant of small size, rapid growth, low chromosome number, and high fertility. Such a plant, *A. thaliana*, was described by Laibach (1943) who provided the initial seed stocks.

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II. MATERIAL

A. thaliana (*Arabis thaliana* L.) belongs to the tribe Arabideae of the Cruciferae. The plant occurs throughout Europe, East Africa, and Northern and Central Asia. A large number of ecotypes, differing chiefly in photoperiod requirement, has been collected and examined by Laibach (1943, 1951).

The seeds when properly stored, retain their full ability to germinate for about 3 years (Laibach, unpublished data). During the present studies it was found that seeds, completely after-ripened, germinated in 2 days at a temperature of 22–26°C and with at least 16 hr of light per day. Such germination also occurred when seeds were taken from the siliqua 12 days after fertilization. Thereafter, the seeds entered upon a dormant period which lasted for about a fortnight after full-ripening. This after-ripening dormancy could be broken by subjecting the seeds to a temperature of 2°C for 8 hr followed by 24°C for 16 hr, and then repeating the cycle. Fully after-ripened seeds usually gave 94–99 per cent. germination.

The photoperiodic response of *A. thaliana* has been examined by Gregory and Hussey (1953). They found that, given 8 hr of daylight each day together with supplementary artificial illumination, the number of days to flowering was progressively reduced the longer the total daily period of light. A further progressive acceleration of flowering was obtained with an increase in the intensity of the supplementary light up to 50 f.c.

When grown in soil, *A. thaliana* may reach a height of 15–35 cm depending upon the ecotype. According to Salisbury (1942) the average seed output of plants growing in the wild is 1650 ± 150 . Plants grown in aseptical culture are much smaller, and their reproductive capacity is lowered accordingly (152 ± 11 seeds per plant). The plant is fully self-fertile, and all ecotypes examined were found to possess a haploid chromosome number of five.

Seed of 10 "races", named after the localities from which the seed was originally collected, were planted under the standard conditions described below in order to select races most suitable for genetical experiments. Of these, the race Enkheim possessed the most rapid development, requiring 13.6 ± 0.3 days from germination to flowering. However, the race Estland which requires 21.9 ± 0.3 days to flower was finally selected because of its vigorous uniform growth.

III. METHODS AND RESULTS

(a) Cultural Conditions

Major mineral requirements were provided as Knop solution and, except for an increased quantity (0.002M) of KH_2PO_4 , at the concentrations recommended by Arnon (1938). The trace elements supplied were iron, manganese, zinc, copper, boron, and molybdenum: iron at 2 p.p.m. and the remainder as used by Arnon (1938). The chemicals were of A.R. grade and the water was glass-distilled.

The mineral solution was adjusted to pH 6.0 approaching the lower ecological pH of *A. thaliana* which, according to Small (1946), is 6.2. During growth, the pH of the medium increased to about 7.1. However, a stable pH obtained by balancing the partial concentration of nitrate against that of ammonia (Trelease and Trelease 1933) did not give significantly better growth.

The nutrient solution was solidified with 0.75 per cent. agar previously purified by extraction with ethanol and pyridine. Besides providing a support for the growing plant, agar is said to make oxygen more accessible (Rippel and Lehmann 1936) and thus the aeration required in liquid cultures is here avoided. The agar concentration is important, for if the gel is not firm enough the leaves become saturated with water and distorted growth results. If the agar is too firm, however, there is variation between plants in the penetration of their primary roots and these delays are carried throughout their subsequent development.

The melted medium was dispensed in 5-ml aliquots to 16 by 150 mm "Pyrex" test tubes, which were then plugged with non-absorbent cotton wool and autoclaved at 15 lb pressure for 15 min. Sometimes the seed production may be much reduced and this effect can generally be attributed to pollen abortion because of an inefficient gas exchange to the outer atmosphere; cotton-wool plugs, therefore, should be quite loose.

The seeds were sterilized by immersion in a solution of absolute ethanol and 20 volumes hydrogen peroxide (1 : 1) for 10 min (Harris, unpublished data), and planted with a platinum loop inside a sterilized cabinet. Seeds may be transferred from this sterilizing solution directly to the agar without there being any subsequent toxic effects. When planting, the seeds should be laid on the surface of the agar; if they are even slightly below the surface the plant may fail to orient itself properly.

Immediately after planting, the cultures were placed at a low temperature (0–5°C) for 24 hr to ensure uniform germination. They were then transferred to a growth chamber which was maintained at 25°C and supplied with continuous illumination of about 800 f.c. intensity from fluorescent light tubes. It was found advantageous to keep the relative humidity above 60 per cent. to prevent the agar medium from drying out too rapidly.

A satisfactory indication of growth rate was obtained by noting the daily increase in length of the primary root, the number of secondary roots, and the date of appearance of successive leaves, floral initials, and the first open flower. In Figures 1, 3, 4, and 5 the mean values and standard deviations (shown by vertical lines) are based on 12–15 plants per treatment.

(b) *Factors Affecting Growth*

(i) *Trace Elements*.—The medium first used consisted only of an aqueous solution of major and trace elements solidified with agar. The plants on this medium, however, rapidly became chlorotic, showed little root growth or leaf expansion, and usually died before setting seed. The general appearance of the plants suggested a trace element deficiency which was confirmed by the finding that the plants were bright green and grew vigorously with the addition of a solution of Seitz-filtered trace elements. Increasing the concentration of iron from 1 to 2 p.p.m. ensured better growth and survival but there was still marked chlorosis during vegetative growth, although the leaves turned green at the time of flowering. Supplying the iron in a chelated form as ferric citrate gave no improvement over ferric sulphate; the mean dry weight at flowering with ferric citrate was 6.0 ± 0.3 mg, and with ferric sulphate, 6.4 ± 0.3 mg.

When ethylenediaminetetra-acetate (EDTA) was used (according to the method of Jacobson 1951) to chelate the ions of copper, zinc, and manganese as well as iron, growth was equal to that obtained with Seitz-filtered trace elements (Fig. 1).

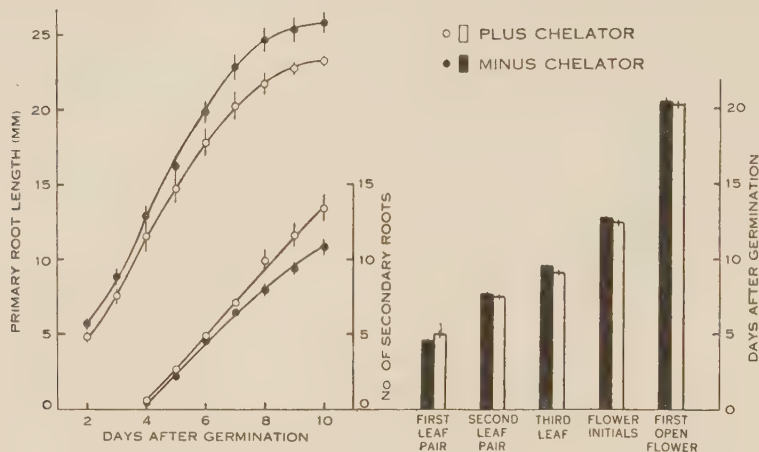


Fig. 1.—Effect of trace element chelation on growth.

Chelation with EDTA resulted in greater secondary root formation, increased vegetative development, and increased dry weight: with EDTA, 8.1 ± 0.3 mg, and without EDTA, 6.4 ± 0.3 mg.



Fig. 2.—Wooden block holding test-tube cultures of 48 plants, 26 days old.

(ii) *Exposure of the Root System to Light.*—In order to obtain optimal growth it was found necessary to shield the root systems of the growing plants from direct light. This was best accomplished by placing the test tubes in holes bored in wooden blocks so that the surface of the agar medium was level with that of the wood (Fig. 2).

The primary effect of light upon root growth was a general decrease in meristematic activity as reflected in shorter primary and fewer secondary roots

(Fig. 3). This inhibition of root growth in turn delayed the formation of floral primordia (by 1 day) and the onset of flowering (by 1.5 days), and also significantly decreased the total dry weight at flowering from the normal 8.1 ± 0.3 mg to 6.9 ± 0.5 mg.

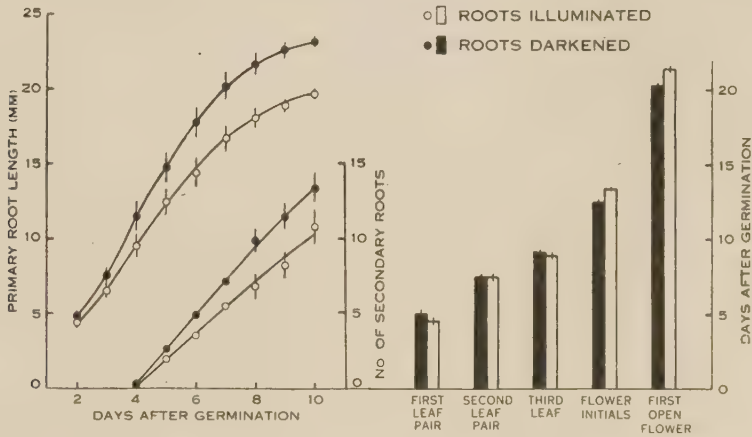


Fig. 3.—Effect on growth of exposing the root system to light.

(iii) *Sucrose*.—As the intensity of light available to the plants is less than the 800 f.c. reaching the test tubes, it was thought advisable to supplement the photo-synthetic supply of carbohydrate. To determine the most readily utilizable sugar, a chlorophyll-deficient mutant, which possessed otherwise normal chloroplasts, was supplied with a range of carbohydrates usually at the rate of 100 mg/plant.

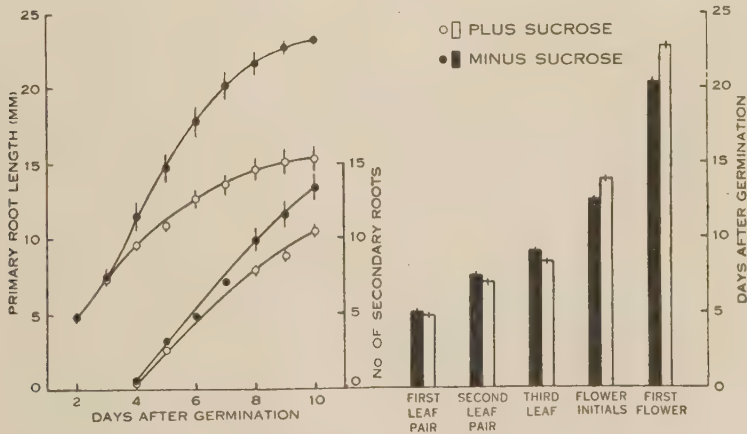


Fig. 4.—Effect of 2 per cent. sucrose on growth.

Sucrose gave the best growth, with glucose only slightly inferior; fructose was a very much poorer carbon source. Although glucose was utilized, there was no growth with glucose phosphates or with other phosphorylated sugars. Sucrose, purified by boiling with activated charcoal, significantly accelerated vegetative development (Fig. 4) and increased the plant mean dry weight from 8.1 ± 0.3 mg to 9.4 ± 0.2 mg.

At the same time, however, it depressed root growth and delayed flowering by 2.5 days.

After the plants had finished growing, the agar from 50 test tubes was examined for substances excreted by the plants during growth. This agar contained large quantities of a carbohydrate which was identified by the melting point of its osazone and by its chromatographic behaviour as fructose. Further chromatographic analysis of the agar at intervals during plant growth showed that although the fructose

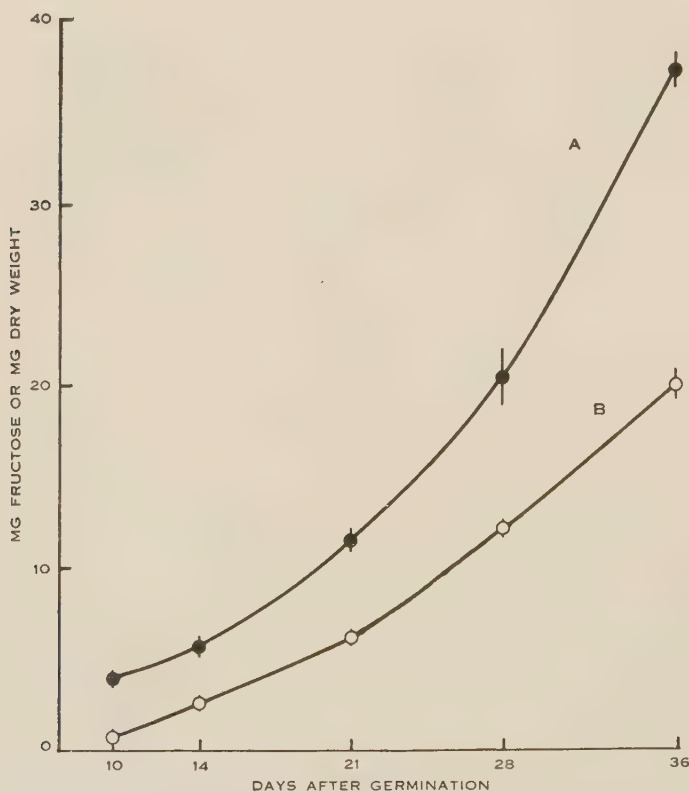


Fig. 5.—Rate of fructose formation (or glucose uptake) in mg/plant (A), and the corresponding plant dry weights (B).

increased in amount, no glucose was to be found. It was concluded that the plants had hydrolysed the sucrose provided in the medium, absorbed the glucose, and left the fructose behind.

The rate of glucose uptake was then followed by quantitative determination of the residual fructose by the Hagedorn-Jensen method for reducing sugar. The curves for fructose production, which may be taken as representing glucose uptake, and the corresponding dry weights of the whole plants are shown in Figure 5. At the time of seed formation each plant was absorbing about 2 mg of glucose per day.

(c) Large-scale Culture

In a segregating progeny only one or two plants in 14 are mutants because of the chimerical nature of the parent plants which develop from irradiated seed, and thus the isolation of mutants by individual plant culture is quite laborious. Therefore, attention was turned to finding methods of growing the plants in bulk culture.

Agar is unsuitable as a medium for large-scale culture because it must be darkened with charcoal to protect the plant roots from light and this leads to heating of the medium and excessive water loss from the agar surface. Many other substrates were tested, and two of these, cellulose pulp and silica gel, proved to be reasonably satisfactory. A cellulose pulp medium is the more easily prepared, but it is readily metabolized by fungi, and different samples of what is supposed to be the same grade of cellulose pulp differ widely in their suitability for plant growth. Silica gel, on the other hand, may be prepared in such a way as to give a standard product, and contamination with microorganisms is not a serious problem.

The method of silica gel preparation was adapted from the work of Daiber (unpublished data, 1944). The silicic acid sol is prepared as follows: 12.5 ml of liquid sodium silicate is diluted with 8.4 ml of glass-distilled water. This solution is added slowly and with cooling to 31 ml of concentrated hydrochloric acid (sp. gr. 1.18). It is necessary to use distilled water, for impurities may cause premature opalescence or coagulation of the sol during dialysis. The silicic acid sol must be dialysed to remove sodium chloride and excess acid and the dialysis should be carried out at a temperature below about 16°C to avoid gel formation. The solution was electrodialysed, using a current of 10 mA, in a cold room for about 24 hr. By this time the resistance of the solution had increased from less than 1 ohm to a constant value of about 50 ohms. 75 ml of the dialysed sol was then mixed with 40 ml of triple-strength mineral nutrient solution, the pH adjusted to 6.0, and 75 ml of the sol was placed in each crystallizing dish (9 by 5 cm). The solution gels in about 1 hr at room temperature, the rate of gelling depending on the pH, salt content, and age of the sol. The dishes and contents were sterilized by steaming for 30 min and planted with sterilized seed in the usual manner. Fifty seeds may be planted in each of 12 dishes in 1 hr.

To keep the cultures aseptic, the space about the lid of the crystallizing dish was packed with cotton wool pre-treated with cetyl pyridinium chloride (0.1 per cent. chloroform solution). The inner surfaces of the lids may be treated with "Anti-dim" to prevent water condensing in large drops and reducing the available light. When the flower stalks reached the lids the plants were about to flower and the lids were then removed to allow flowering and seed-setting to take place normally. Fungal contamination at this stage of the plants' growth did not affect their further development.

Growth was more variable on silica gel than on agar mainly because of the greater occurrence of delayed root penetration with the former substrate. This disadvantage, however, was offset by the much larger populations which could be screened for mutants.

(d) *The Excretion of Organic Substances*

A possible objection to the use of bulk-culture methods for the isolation of growth mutants is that cross-feeding between normal and mutant plants may occur, thus preventing the detection of certain types of mutants. Accordingly, a study was made of the excretions from the roots of wild-type *A. thaliana* when grown in agar.

Plants were grown to maturity on agar medium to which 2 per cent. purified sucrose had been added on the assumption that a high carbohydrate level in the plant might increase the amount of excretion. All plant material was removed from the agar which was then examined for vitamins, amino acids, and reducing substances.

The vitamin assays were carried out using vitamin-requiring mutants of *Neurospora crassa*. The agar samples to be tested were inoculated in duplicate with conidia from a given mutant and incubated at 24°C for 5 days. Wild-type *Neurospora* grew well on aliquots of the agar with no additions, this indicating the presence of biotin. Using suitable mutants, qualitative tests were made for eight vitamins of the B group, but activity was found only with respect to pyridoxine, thiamine, and inositol. Pyridoxine and inositol were present in amounts suboptimal for maximum growth of the fungus as shown by a yellowing of the medium on which the pyridoxine-less mutant was grown, and by the colonial growth of the inositol-less mutant.

α -Amino nitrogen in the agar was estimated colorimetrically using ninhydrin, by the method described by Milton and Waters (1949). Although this method is sensitive to 5 μ g of amino nitrogen per ml, no positive reaction was obtained.

The agar was next examined by extracting with ethanol, chromatographing the extracts, and spraying the chromatograms for reducing substances. Only fructose was found, and, after mild acid hydrolysis, fructose and glucose from the residual sucrose were found.

A thin layer of white material appeared on the surface of the agar near the plant stem at about the time the plants flowered. This wax-like substance was very insoluble and was not further examined. As the plants grew, the medium showed a blue fluorescence in ultraviolet light which deepened as growth continued. However, all attempts to isolate the fluorescent substance were unsuccessful.

These tests appear to indicate that wild-type plants do not excrete sufficient material to interfere with the detection of growth mutants when segregating plants are grown together.

IV. DISCUSSION

A small rapidly growing plant such as *A. thaliana* offers many advantages for experimental work on flowering plants. It is especially suitable for mutation and other genetic studies because of its short life cycle, few chromosomes, and high seed production. These advantages are enhanced by the relative ease with which

plants may be maintained under sterile conditions. The consequent precision of environmental control facilitates physiological experiments on growth and development, the addition of organic substances to the substrate, and the study of gene-environment interactions. In addition, the small seeds with their low nutrient reserves make the plants very sensitive to mineral deficiencies, and thus potentially valuable for mineral bioassay. A brief report of the use of *A. thaliana* in the production and isolation of biochemical mutations has recently appeared (Langridge 1955).

The critical factors for optimal growth in aseptie culture are chelation of trace element cations and protection of the root system from direct light. Chelation is presumably necessary to avoid precipitation of cations during autoclaving of the medium. The continued growth of excised tomato roots in culture has similarly been shown to be limited by an unavailability of iron which may be overcome by the addition of chelated iron (Street, McGonagle, and McGregor 1952). According to Weinstein, Robbins, and Perkins (1954) the complexed iron is not only more readily absorbed by plant roots, but is also maintained in a form which is more readily metabolized by the plant.

The mode of action of light in inhibiting root meristem activity, with a consequent lowering of total dry weight, is not known with certainty. It has been shown by Naundorf (1940) and Pilet (1953) that light causes changes in the auxin concentration of roots. However, the former author found that illuminated roots of *Helianthus* produced more auxin than non-illuminated ones, while the latter stated that light caused an inactivation of auxin in *Lens* roots. Although indolylacetic acid was supplied in a range of concentrations to light-exposed roots of *A. thaliana*, it failed to increase root growth or dry weight.

The ability of *A. thaliana* to utilize different sugars is similar to that reported for other plants (e.g. Allsopp 1953). Sucrose and glucose are about equal in value as carbon sources, fructose is only slowly utilized, and most other sugars are either quite inferior or toxic. Dormer and Street (1949) have found that excised tomato roots will grow on sucrose, but not on glucose, fructose, or an equimolecular mixture of the two. They suggest that this is because carbohydrate can only be absorbed as hexose phosphate and that hydrolysis of sucrose is necessary to provide the energy for phosphorylation. Although roots of *A. thaliana* can hydrolyse sucrose either by a mechanism operating at the root surface or by the secretion of an enzyme into the substrate, there is no evidence that energy available from hydrolysis is used in phosphorylation of the glucose. The fact that free glucose supports as good a growth of albino mutants as does sucrose suggests that external phosphorylation is not essential for absorption by the intact plant.

V. ACKNOWLEDGMENTS

I wish to thank Professor D. G. Catcheside, formerly Professor of Genetics, University of Adelaide, for his helpful advice; Professor Emeritus F. Laibach of Frankfurt-on-Main for seeds of *A. thaliana*; Mr. C. G. Greenham, Division of Plant Industry, C.S.I.R.O., for help with the electrodialysis; and the Commonwealth Scientific and Industrial Research Organization for the award of a studentship.

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NUTRIENT INTERACTIONS AND DEFICIENCY DIAGNOSIS IN THE LETTUCE

III. NITROGEN CONTENT AND RESPONSE TO NITROGEN

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[*Manuscript received August 30, 1956*]

Summary

Lettuce plants grown in sand culture, and receiving nitrogen, phosphorus, and potassium at five levels in all combinations, were analysed at different stages of growth for total, soluble, and nitrate nitrogen. An attempt was made to relate these analytical data to the subsequent response (in dry matter production) shown by the plants when a further amount of nitrogen was supplied.

The percentage of total nitrogen in the plant dry matter was generally increased by increasing levels of nitrogen or phosphorus supply, and by potassium deficiency. The increase in nitrogen content with phosphorus supply depended on the level of the other nutrients, being most marked where nitrogen supply was high, or potassium low. Changes in content of nitrate nitrogen were in general similar to those for total nitrogen, but much more marked.

The increase in dry weight of the plants as a result of supplementary nitrogen, supplied at 46 days from sowing, was closely related to the nitrogen and phosphorus content of the plants at earlier stages, not at all to their potassium content. Where total nitrogen content was used, the best forecast of response was obtained by sampling immediately before the supplementary nitrogen was applied; where nitrate analyses were used, the optimum time for sampling was two weeks earlier. In each case, data for phosphorus content provided valuable additional information as to the response potentialities of the plants. Determination of insoluble, or of total soluble nitrogen gave no advantage over total or nitrate nitrogen respectively. No clear preference could be expressed between older and younger leaves as sources of material for analysis, and nitrate determinations on midribs were disappointing. The relationship of response to the content of nitrogen and phosphorus could be fitted better by a curved than a plane surface.

I. INTRODUCTION

The principal purpose of the investigation described in the present series of papers was to study the relationship between the growth responses to nutrients applied to half-grown lettuce plants and the composition of the same plants at the time of application or earlier. Lettuce plants differing widely in nutritional status were obtained by sand-culture methods; five levels each of nitrogen, phosphorus, and potassium supply in all combinations were used. Samples of plants from some or all treatments were taken for analysis at 11, 22, 29, 37, and 44 days from sowing. At 46 days, additional amounts of nitrogen, phosphorus, or potassium were supplied each to one-quarter of the plants, while the rest received no further treatment. All plants were finally harvested at 98 days.

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Apart from the main purpose of the investigation, the experiment also provided abundant information on the effects of nutritional interactions on the growth and composition of the plants. The first two papers (Goodall, Grant Lipp, and Slater 1955; Goodall, Slater, and Grant Lipp 1957) thus dealt with the results in respect of dry matter and water content. The present paper presents the data for nitrogen content, and will then proceed to discuss the relationship between the responses to the nitrogen application at 46 days and the previous composition of the plants.

II. MATERIALS AND METHODS

Details of the sand-culture and harvesting procedure have been given in the first paper of this series (Goodall, Grant Lipp, and Slater 1955).

For all the chemical analyses, the dry material was finely ground in a mortar, and portions of the powder were dried again at 100°C before analysis. The small amount of material available from many plants made it necessary to use semi-micro methods of analysis, and also prevented the determination of various nitrogen fractions which might have been of interest. Besides the total nitrogen content, it was possible in most samples also to determine the insoluble, soluble, and nitrate nitrogen. The analytical data are expressed as percentages of dry matter, except for nitrate nitrogen, which is given as parts per million.

Total nitrogen was determined by a form of the Kjeldahl method, described in detail by Slater (1952). The method was checked by frequent analysis of acetanilide: a consistent accuracy of 98–101 per cent. recovery of nitrogen was found, with duplicate determinations agreeing within 1 per cent.

Soluble nitrogen was extracted by shaking with water for 30 min—a procedure which gave similar values for insoluble nitrogen to those obtained by extracting with trichloroacetic acid, and did not (like the latter) cause loss of nitrate. Nitrate and total soluble nitrogen were both determined in the aqueous extract, the former by the phenoldisulphonic acid method (Ulrich 1948), the latter by the Kjeldahl method; details of procedure are given by Slater (1952).

Total nitrogen was determined on plants from 27 treatments in the 11- and 22-day harvests, the levels 2 and 4 of nitrogen, phosphorus, and potassium being omitted (see Goodall, Grant Lipp, and Slater 1955, p. 305). In the 29-, 37-, and 44-day harvests the analytical labour was reduced by omitting those treatment levels which seemed least likely to yield useful information (viz. N_4 and K_4). Replicate samples of plant material were combined for analysis. All available plants from the 98-day harvest were analysed, but so many plants receiving phosphorus levels P_1 and P_2 died before reaching this stage that it was necessary to omit these levels completely from the analysis of variance.

It will be recalled that, in the larger plants, the aerial parts harvested had been separated into younger and older leaves, and the latter further divided into midribs and laminae. These groups of organs were analysed separately. The data were too incomplete to provide a satisfactory basis for assessing statistically the effects of the treatments and their interactions, but were studied as a possible basis for estimating response.

For reasons explained before (Goodall, Grant Lipp, and Slater 1955) certain plants died before harvest. In a few other instances the amount of material was insufficient for chemical analysis. In all these cases "missing plot" values had to be fitted before proceeding to analyses of variance. The methods used are described by Slater (1952). One degree of freedom was subtracted for each fitted value in the analyses of variance.

III. RESULTS

Table 1 presents analyses of variance for the per cent. total nitrogen in the plants at each harvest, including those harvested at 98 days and receiving no sub-treatment. The analyses of the nitrate data for earlier harvests are also presented

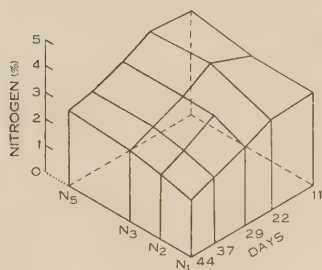


Fig. 1

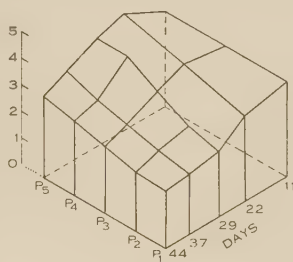


Fig. 2

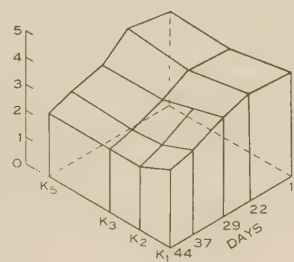


Fig. 3

Figs. 1-3.—Effect of nitrogen (Fig. 1), phosphorus (Fig. 2), and potassium (Fig. 3) supply on total nitrogen content at different stages of development.

in this table; nitrate analyses were not performed on the 98-day harvest. Table 2 gives analyses of variance for the effects of the three sub-treatments on nitrogen content at 98 days. Similar tables are not given for the effects of initial treatments and sub-treatments on soluble and insoluble nitrogen content, because their variation closely paralleled that in nitrate and total nitrogen respectively. The mean values of nitrogen and nitrate content corresponding with effects shown to be significant in the analyses of variance are presented in Tables 3-8, and, in appropriate cases, solid diagrams have been prepared (Figs. 1-10). Fuller data are available in the original thesis (Slater 1952) on which this paper is based.

(a) Nitrogen Content as Affected by Initial Treatments

(i) *Total Nitrogen.*—The total nitrogen content was highest in the young plants, and fell progressively as they grew (see Figs. 1-3). The data quoted for 11 days refer to the whole plants, and the presence of the roots may have been responsible for the fact that the nitrogen content at this time was lower than for the harvest at 22 days. As the solid diagrams show, the decline in nitrogen content with age was largely independent of treatment.

The nitrogen content of the plants at all stages of development increased with initial nitrogen supply (Fig. 1). But such effects were recorded for the higher levels of nitrogen supply only at the later harvests (if one excepts the anomalous results at 11 days). At 29 days, the maximum nitrogen content was already reached

in the N_2 treatments, and at 37 days in the N_3 treatments; only from 44 days did the increase continue up to N_5 . Since yield reached a maximum at N_3 , these results represent marked "luxury consumption" at N_5 . Phosphorus supply had the effect of increasing nitrogen content throughout the range tested, from the second harvest onwards (Fig. 2); the decrease in nitrogen content with time was more marked at higher than at lower levels of phosphorus supply. Potassium, unlike phosphorus, had the effect of decreasing nitrogen content (Fig. 3); this was, however, true only of the lowest potassium supply given (K_2)—higher levels of supply had no further effect.

Of the interactions which were significant when compared with the second-order interaction, that between nitrogen and phosphorus was the most consistent, being evident at each harvest (Fig. 4). The phosphorus-potassium interaction (Fig. 5) was significant only at 29, 37, and 44 days, and the nitrogen-potassium interaction not at all.

The general effect of the interactions between nitrogen and phosphorus supply (Fig. 4) was that the increase in nitrogen content with increased supply was much

TABLE I
NITROGEN CONTENT AS AFFECTED BY INITIAL TREATMENTS: ANALYSES OF VARIANCE

Days from Sowing	Source of Variation	Degrees of Freedom	Mean Square	
			Total Nitrogen (% of dry matter)	Nitrate Nitrogen (p.p.m. of dry matter)†
11	N	2	0.799**	0.9200*
	P	2	0.003	0.0031
	K	2	0.596**	0.0013
	$N \times P$	4	0.524**	0.0300
	$N \times K$	4	0.077	0.0136
	$P \times K$	4	0.135	0.0615
	Error	7	0.054	0.1031
22	N	2	1.571***	1.6922***
	P	2	3.059***	0.3342***
	K	2	0.375*	0.0392*
	$N \times P$	4	0.350*	0.0762**
	$N \times K$	4	0.061	0.0219
	$P \times K$	4	0.137	0.0164
	Error	8	0.066	0.0073
29	N	3	1.534***	2.3917***
	P	4	8.711***	0.9052***
	K	3	1.480***	0.3276***
	$N \times P$	12	0.297**	0.0956*
	$N \times K$	9	0.073	0.0342
	$P \times K$	12	0.251**	0.0602
	Error	36	0.075	0.0365

TABLE 1 (*Continued*)

Days from Sowing	Source of Variation	Degrees of Freedom	Mean Square	
			Total Nitrogen (% of dry matter)	Nitrate Nitrogen (p.p.m. of dry matter)†
37	N	3	1.144***	1.6076***
	P	4	3.543***	0.1791
	K	3	1.225***	0.4298**
	N × P	12	0.399**	0.1071
	N × K	9	0.136	0.0611
	P × K	12	0.348*	0.2159**
	Error	36	0.139	0.0709
44	N	3	2.058***	2.6257***
	P	4	2.097***	0.2101**
	K	3	1.903***	0.3633***
	N × P	12	0.484**	0.0777
	N × K	9	0.173	0.0468
	P × K	12	0.295*	0.0709
	Error	36	0.121	0.0467
98	N	4	18.991***	n.d.
	P	2	1.974**	
	K	4	1.935***	
	N × P	8	0.660*	
	N × K	16	0.266	
	P × K	8	0.115	
	Error	28	0.249	

* $P=0.01-0.05$.** $P=0.001-0.01$.*** $P<0.001$.

† Logarithmic transformation.

more evident at high than at low levels of phosphorus—though even at P_1 the effect was recognizable. This applied to all harvests except the first, where the aberrant figure for N_3P_1 was responsible for the significant interaction. Consequently the increase in nitrogen content with increasing phosphorus supply became more marked at higher nitrogen levels.

The interactions between phosphorus and potassium supply are illustrated in Figure 5. The principal feature was that the increase in nitrogen content with increasing phosphorus supply was more marked at the lowest potassium levels than elsewhere, i.e. the decline in nitrogen content with potassium supply was hardly evident at low phosphorus supply, but became clearer as the phosphorus supply increased.

(ii) *Nitrate Nitrogen*.—The general picture for nitrate nitrogen was similar to that for total nitrogen, but there were several differences. The most striking was the far wider range of values, the lowest nitrate contents being about 150 p.p.m.

and the highest 15,000 p.p.m.; this wide range made a logarithmic transformation necessary before analysis of variance. The overall mean nitrate content fell sharply from 22 days to 37 days, but not thereafter (Figs. 6–8). The mean content at 11 days was less than half that at 22 days—a difference which can hardly be attributed to

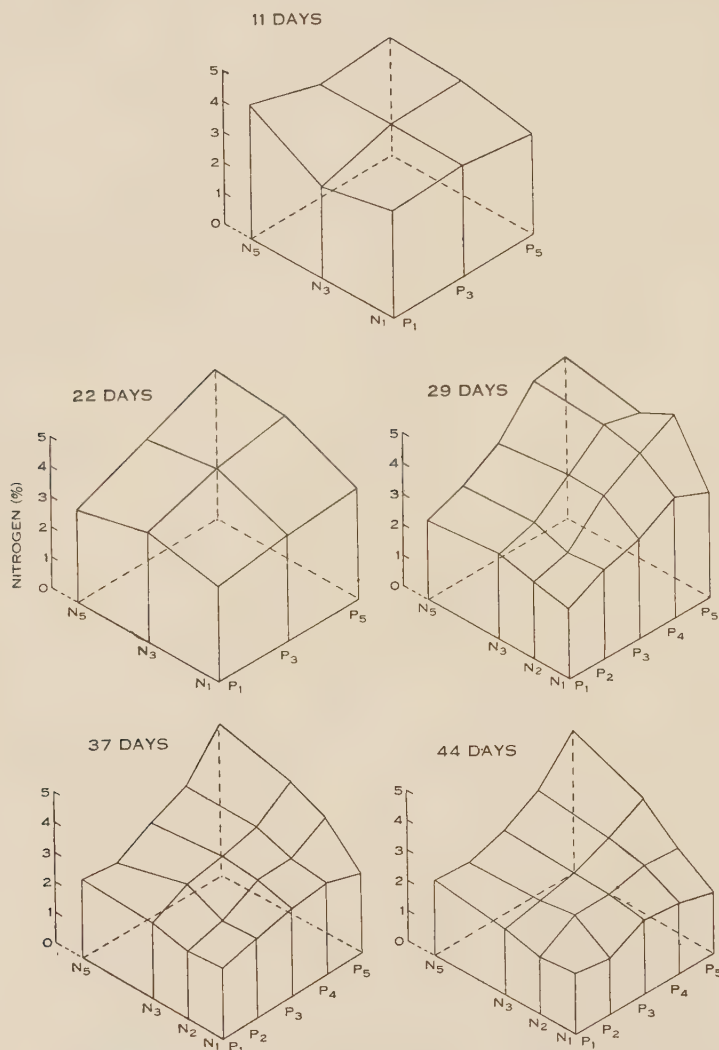


Fig. 4.—Interaction effects of nitrogen and phosphorus supply on total nitrogen content.

the presence of the roots in the former, but rather to the fact that, as shown below, uptake from the culture medium had barely begun at that stage.

The initial supply of nitrogen was consistently reflected in the nitrate content of the plants (Fig. 6), at all levels and at all stages of development. Increases in phosphorus supply, too, consistently caused increases in nitrate content from 22 days

onwards (Fig. 7). Potassium supply decreased nitrate content (Fig. 8) as with total nitrogen, but no noticeable differences occurred among the various levels (K_2 – K_5) of potassium supply.

As with total nitrogen, only the $N \times P$ and $P \times K$ interactions were significant. The interaction between nitrogen and phosphorus, though it reached significance

TABLE 2
TOTAL NITROGEN CONTENT (PER CENT. OF DRY MATTER) AT 98 DAYS
AS AFFECTED BY SUB-TREATMENTS: ANALYSIS OF VARIANCE

	Initial Treatments	Degrees of Freedom	Mean Square
Nitrogen sub- treatment effect		1	3.582***
Interaction with	N	4	1.361***
	P	2	0.048
	K	4	0.347*
	$N \times P$	8	0.200
	$N \times K$	16	0.286***
	$P \times K$	8	0.395***
Phosphorus sub- treatment effect		1	0.019
Interaction with	N	4	0.726***
	P	2	0.643**
	K	4	0.223
	$N \times P$	8	0.157
	$N \times K$	16	0.197*
	$P \times K$	8	0.462***
Potassium sub- treatment effect		1	1.653***
Interaction with	N	4	0.048
	P	2	1.628***
	K	4	1.472***
	$N \times P$	8	0.356***
	$N \times K$	16	0.305***
	$P \times K$	8	0.371***
Error		276	0.104

* $P=0.01$ – 0.05 .

** $P=0.001$ – 0.01 .

*** $P<0.001$.

only in the plants harvested at 22 and 29 days, showed a similar trend at 37 and 44 days too (Fig. 9). The increase in nitrate content with increasing phosphorus supply was much more marked at higher than at lower levels of nitrogen supply—indeed, at N_1 it was negligible.

As for the interaction between phosphorus and potassium (Fig. 10), this too was similar at all harvests after 11 days, though significant only at 37 days. It consisted

in the fact that the decrease in nitrate content with potassium supply mentioned above was marked only at the higher levels of phosphorus supply.

(b) *Effects of Sub-treatments on Nitrogen Content*

Since nitrate analyses were not performed on the 98-day harvest, the effects of sub-treatments could only be studied on the total nitrogen content; as already mentioned, treatments including P_1 and P_2 were omitted from the statistical analysis because so many data for them were missing. The results of analysis of variance are presented in Table 2, the error term being obtained from the highest-order interaction for all sub-treatments taken together, whereas the interaction terms

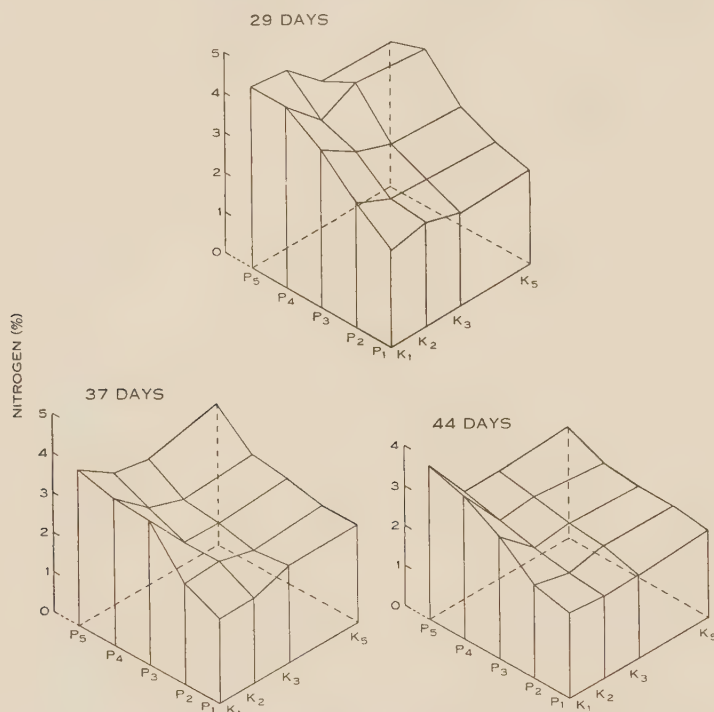


Fig. 5.—Interaction effects of phosphorus and potassium supply on total nitrogen content.

for each sub-treatment with the various initial treatments have been computed separately. The mean effects of the sub-treatments are shown in Tables 6–8, being expressed as the difference in total nitrogen content (per cent. of dry matter) between plants receiving the sub-treatment in question and plants receiving no additional nutrient supply.

(i) *Nitrogen Sub-treatment*.—The average effect of the nitrogen sub-treatment was to increase the nitrogen content of the plant by 0.31 per cent. This effect was more marked after low initial nitrogen supply, and negligible after high levels; it was unaffected by the initial supply of phosphorus (between the levels of P_3 and P_5). The initial supply of potassium affected the increase in nitrogen content as a result of

supplementary nitrogen supply, in that this increase was substantial at low and high levels of initial potassium and smaller or negligible at intermediate levels: the large increase under conditions of low potassium supply might be explained as "luxury consumption" when growth was limited by the potassium level, but the differences between K_3 , K_4 , and K_5 are more difficult to account for.

TABLE 3

TOTAL NITROGEN CONTENT (PER CENT. OF DRY MATTER) AS AFFECTED BY NITROGEN AND PHOSPHORUS SUPPLY

Days from Sowing	Nitrogen Levels	Phosphorus Levels				
		P ₁	P ₂	P ₃	P ₄	P ₅
11	N ₁	3.47	—	3.58	—	3.21
	N ₃	3.00	—	3.68	—	3.66
	N ₅	4.38	—	3.66	—	3.85
22	N ₁	3.11	—	3.44	—	3.63
	N ₃	3.58	—	4.32	—	4.68
	N ₅	3.05	—	4.01	—	4.92
29	N ₁	2.25	2.73	2.98	3.49	3.14
	N ₂	2.44	2.65	3.53	4.07	4.55
	N ₃	2.59	2.88	3.53	4.30	4.06
	N ₅	2.48	2.79	3.33	4.37	4.59
37	N ₁	2.21	2.44	2.69	2.79	2.44
	N ₂	2.14	2.40	2.88	2.91	3.42
	N ₃	2.34	2.78	2.98	3.19	3.82
	N ₅	2.48	2.34	2.81	3.21	4.26
44	N ₁	2.02	1.92	2.36	2.28	2.03
	N ₂	1.94	2.48	2.52	2.71	2.59
	N ₃	2.11	2.31	2.49	2.89	3.38
	N ₅	2.39	2.30	2.59	3.08	4.04
98	N ₁	—	—	1.97	1.92	1.55
	N ₂	—	—	2.45	2.53	2.50
	N ₃	—	—	2.66	3.30	3.32
	N ₄	—	—	3.11	4.14	4.48
	N ₅	—	—	4.66	4.71	5.10

Of the second-order interactions of nitrogen sub-treatment with initial treatments, that with initial nitrogen and phosphorus approaches significance, and consists in a tendency for the sub-treatment to decrease nitrogen content where initial nitrogen and phosphorus supply were high, while increasing it under other circumstances. The other second-order interactions are highly significant but rather obscure. The interaction with initial nitrogen and potassium seems to be

attributable to the peculiar high positive value at N_5K_5 ; this resulted from a single very small plant with very high nitrogen content, and so may perhaps be ignored as fortuitous. In the interaction with initial potassium and phosphorus the most obvious element is the fact that the maximum effects of the sub-treatment on nitrogen

TABLE 4
TOTAL NITROGEN CONTENT (PER CENT. OF DRY MATTER) AS AFFECTED BY PHOSPHORUS AND POTASSIUM SUPPLY

Days from Sowing	Potassium Levels	Phosphorus Levels				
		P ₁	P ₂	P ₃	P ₄	P ₅
29	K ₁	2.43	3.08	3.93	4.53	4.53
	K ₂	2.59	2.71	3.41	3.69	4.44
	K ₃	2.34	2.68	3.04	4.10	3.65
	K ₅	2.39	2.57	3.00	3.92	3.61
37	K ₁	2.12	2.57	3.62	3.71	3.95
	K ₂	2.15	2.59	2.55	2.96	3.32
	K ₃	2.47	2.35	2.55	2.67	3.20
	K ₅	2.44	2.46	2.64	2.75	3.52
44	K ₁	2.14	2.32	3.03	3.57	3.84
	K ₂	2.05	2.11	2.27	2.48	2.69
	K ₃	2.07	2.30	2.37	2.55	2.72
	K ₅	2.20	2.29	2.28	2.37	2.78

content occurred when the supply of either potassium or phosphorus was high, while the other was low. This would suggest that the nitrogen content is most sensitive to changes in supply when growth is limited by lack of one other nutrient only.

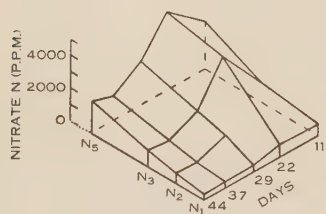


Fig. 6

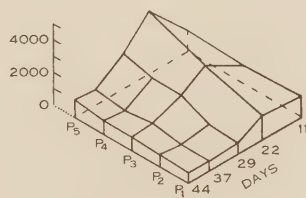


Fig. 7

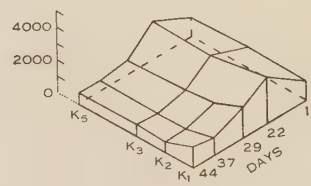


Fig. 8

Figs. 6-8.—Effect of nitrogen (Fig. 6), phosphorus (Fig. 7), and potassium (Fig. 8) supply on nitrate nitrogen content at different stages of development.

(ii) *Phosphorus Sub-treatment.*—The average effect of phosphorus sub-treatment on nitrogen content was negligible (-0.02 per cent.), but some of its interactions with the initial treatments were highly significant. At high initial nitrogen, phosphorus supplements increased the nitrogen content; high initial phosphorus, on

the other hand (as might be expected), reduced the response of nitrogen content to added phosphorus.

The second-order interaction between phosphorus sub-treatment and nitrogen and potassium initial treatments was obscure and only just significant; it may probably be safely ignored. That with initial potassium and phosphorus supply was highly significant, and only in part accounted for by the aberrant value

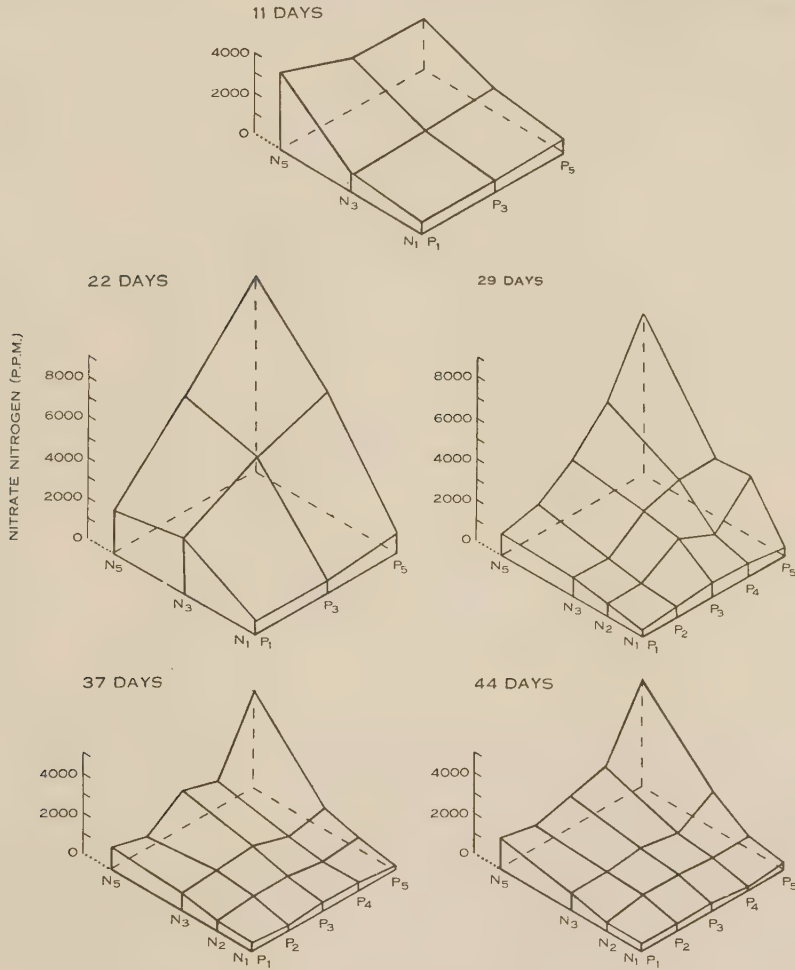


Fig. 9.—Interaction effects of nitrogen and phosphorus supply on nitrate nitrogen content.

for K_1P_3 ; it seems to have consisted in a reduction in the effect of initial phosphorus on the response (in nitrogen content) to phosphorus supplements at intermediate levels of potassium.

(iii) *Potassium Sub-treatment*.—The average effect of the potassium sub-treatment was to reduce the nitrogen content by 0.21 per cent. This was not dependent on initial nitrogen supply. Where potassium was supplied initially (K_2-K_5) the effect

was negligible, but was very marked where the plants had been deprived of potassium; it was more noteworthy at higher than lower initial levels of phosphorus.

TABLE 5

NITRATE NITROGEN CONTENT (P.P.M. OF DRY MATTER) AS AFFECTED BY INITIAL NUTRIENT SUPPLY:
GEOMETRIC MEANS

11 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

N ₁	668	P ₁	1268	K ₁	1156
N ₃	1023	P ₃	1183	K ₃	1279
N ₅	2802	P ₅	1278	K ₅	1238

22 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

N ₁	796	P ₁	1592	K ₁	2988
N ₃	4172	P ₃	2592	K ₃	2243
N ₅	4797	P ₅	3861	K ₅	2374

Interactions between Initial Treatments

	N ₁	N ₃	N ₅
P ₁	766	2624	2008
P ₃	662	4642	5649
P ₅	996	5957	9698

29 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

N ₁	413	P ₁	648	K ₁	1811
N ₂	1201	P ₂	721	K ₂	1133
N ₃	1676	P ₃	1597	K ₃	1065
		P ₄	1659		
N ₅	2697	P ₅	2215	K ₅	984

Interactions between Initial Treatments

	N ₁	N ₂	N ₃	N ₅
P ₁	293	642	914	1022
P ₂	349	678	806	1419
P ₃	577	1803	2231	2797
P ₄	551	1095	2814	4467
P ₅	369	2907	2857	7852

TABLE 5 (*Continued*)

37 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

N ₁	331	P ₁	574	K ₁	1314
N ₂	550	P ₂	642	K ₂	598
N ₃	887	P ₃	852	K ₃	575
		P ₄	647		
N ₅	1526	P ₅	970	K ₅	604

Interactions between Initial Treatments

	K ₁	K ₂	K ₃	K ₅
P ₁	577	474	653	609
P ₂	734	900	492	523
P ₃	2017	760	433	794
P ₄	1615	603	497	363
P ₅	2835	390	908	882

44 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

N ₁	298	P ₁	574	K ₁	1221
N ₂	647	P ₂	695	K ₂	588
N ₃	1075	P ₃	818	K ₃	719
		P ₄	796		
N ₅	2000	P ₅	1176	K ₅	750

Second-order interactions were also significant. The effect of initial phosphorus on the response to potassium supplementation (mentioned above) was most evident at the higher levels of nitrogen supply—i.e. at N₄ and N₅ the potassium sub-treatment increased nitrogen content at P₃ but decreased it at P₄ and P₅, whereas at lower levels of initial nitrogen supply the phosphorus supply had little effect. The decrease in nitrogen content as a result of the addition of potassium to plants short of this nutrient was particularly marked where the initial nitrogen supply had been abundant. The interaction of potassium sub-treatment with initial potassium and phosphorus supply is obscure and would seem to have little physiological meaning.

(c) Nitrogen Uptake

The analytical data for total nitrogen content and the mean dry weights were combined to determine the total quantity of nitrogen contained in the aerial parts of the plants at each harvest, or, at 11 days, in the whole plants. Only those treatments for which this information was available at each of the six harvests, viz. the levels 1, 3, and 5 of each nutrient, were analysed in this way.

At 11 days the mean amount of nitrogen in plants receiving the N_1 and N_3 treatments was 0.06 mg, the same as that in a single seed, but under the N_5 treatments there had been a slight uptake to give 0.08 mg per plant. Up to 44 days the most rapid uptake occurred in the N_5 treatments, provided phosphorus and potassium levels were favourable. The mean rate of uptake in the N_3 treatments was only a little lower, and increased relatively to that in N_5 during this period; at 98 days all but one of the plants receiving N_3 treatments contained more nitrogen than the corresponding N_5 plants.

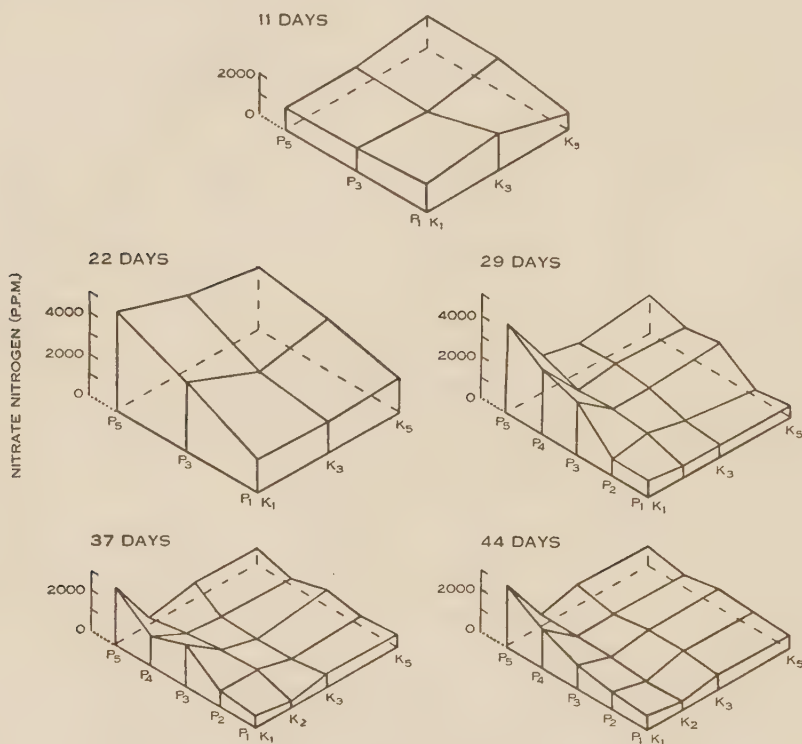


Fig. 10.—Interaction effects of phosphorus and potassium supply on nitrate nitrogen content.

The effects of the three sub-treatments on nitrogen uptake were well-marked. The plants which had been deficient in nitrogen or phosphorus and had been provided with a sub-treatment of the same nutrient recovered to such an extent that their total uptake rose to about half of the maximum figure, while in the potassium-deficient plants the effect of the deficiency was completely abolished by the potassium sub-treatment.

The initial supply of nitrogen to each pot was 50 mg, 500 mg, and 5000 mg in the N_1 , N_3 , and N_5 treatments respectively. The sub-treatment, applied at 46 days, consisted of 200 mg nitrogen. In the N_1 treatments, the total amount of nitrogen contained in the tops at 98 days reached a maximum of 19.4 mg; this represented nearly 40 per cent. of the supply, to which must be added an (unknown) amount

in the roots, and some 5 mg (10 per cent. of supply) for plants removed from the pot at earlier harvests—i.e. more than half the nitrogen supplied was utilized in some of these treatments. At the N_3 level of nitrogen supply, more than 40 per cent. of the amount added was contained in plants harvested at 98 days, in some cases where phosphorus and potassium supply was abundant. So in these cases again more than half the nitrogen supplied would have been used; but at N_5 the

TABLE 6

RESPONSE OF TOTAL NITROGEN CONTENT (PER CENT. OF DRY MATTER) AT 98 DAYS TO NITROGEN SUB-TREATMENT, AS AFFECTED BY INITIAL NUTRIENT SUPPLY

Average Effects at Different Levels of Initial Treatments

N_1	+0.94			K_1	+0.56
N_2	+0.50			K_2	+0.33
N_3	+0.24	P_3	+0.29	K_3	+0.03
N_4	-0.09	P_4	+0.38	K_4	+0.17
N_5	-0.04	P_5	+0.26	K_5	+0.46

Interactions between Initial Treatments

	N_1	N_2	N_3	N_4	N_5
P_3	+0.61	+0.32	+0.12	+0.12	+0.30
P_4	+1.35	+0.63	+0.18	-0.03	-0.24
P_5	+0.87	+0.55	+0.41	-0.37	-0.18
	N_1	N_2	N_3	N_4	N_5
K_1	+1.58	+0.67	+0.37	+0.58	-0.38
K_2	+0.80	+0.32	+0.62	-0.16	+0.08
K_3	+0.91	+0.61	-0.26	-0.29	-0.82
K_4	+0.96	+0.13	+0.23	-0.31	-0.18
K_5	+0.47	+0.77	+0.23	-0.29	+1.10
	K_1	K_2	K_3	K_4	K_5
P_3	+0.04	+0.40	+0.01	-0.09	+1.10
P_4	+0.76	+0.45	+0.25	+0.35	+0.07
P_5	+0.89	+0.14	-0.17	+0.23	+0.19

maximum absorption of nitrogen was less than 4 per cent. of the supply. The proportion of sub-treatment nitrogen absorbed in those treatments where its effect was greatest corresponds closely to the uptake from the initial N_3 treatments—about 100 mg, or 50 per cent.

(d) *Relationship between Growth Response to Nitrogen and Chemical Composition*

The data available on the chemical composition of the plants before the addition of the sub-treatments included the total, protein, non-protein, and nitrate

nitrogen contents of the whole plants on five occasions, as well as less complete data on the composition of the portions, which could be analysed separately only in the larger plants. Data on phosphorus and potassium content were also available, having been determined as described by (Grant Lipp 1952). As explained previously, the plants receiving the N_4 and K_4 treatments were not analysed, so that analytical data were available for 80 treatments at 29, 37, and 44 days. All the plants which

TABLE 7

RESPONSE OF TOTAL NITROGEN CONTENT (PER CENT. OF DRY MATTER) AT 98 DAYS TO PHOSPHORUS SUB-TREATMENT, AS AFFECTED BY INITIAL NUTRIENT SUPPLY

Average Effects at Different Levels of Initial Treatments

N_1	+0.08			K_1	-0.05
N_2	-0.10			K_2	+0.23
N_3	-0.48	P_3	+0.24	K_3	+0.05
N_4	+0.01	P_4	-0.14	K_4	-0.19
N_5	+0.38	P_5	-0.17	K_5	-0.16

Interactions between Initial Treatments

	N_1	N_2	N_3	N_4	N_5
P_3	+0.07	-0.11	-0.08	+0.52	+0.79
P_4	-0.01	-0.20	-0.49	-0.21	+0.21
P_5	+0.18	+0.02	-0.87	-0.29	+0.13
	N_1	N_2	N_3	N_4	N_5
K_1	-0.09	+0.10	-0.83	+0.49	+0.10
K_2	+0.41	-0.33	+0.12	+0.03	+0.60
K_3	+0.08	+0.41	-0.64	+0.01	+0.40
K_4	+0.53	-0.19	-0.94	-0.18	+0.14
K_5	-0.53	-0.37	-0.32	-0.32	+0.66
	K_1	K_2	K_3	K_4	K_5
P_3	+0.81	+0.31	-0.23	-0.10	+0.40
P_4	-0.74	+0.39	+0.28	-0.09	-0.54
P_5	-0.21	+0.01	+0.10	-0.40	-0.34

had been harvested at 11 and 22 days were analysed, giving data for 27 treatments on each of these occasions.

The response associated with each treatment was given by the difference between the dry weights for the nitrogen sub-treatment and its control at 98 days, the differences being corrected for differences in size at the time of applying the sub-treatment, as described earlier (Goodall, Grant Lipp, and Slater 1955). The responses, expressed in milligrams, are shown in Table 9.

The relationship between the responses measured in this way and the nitrogen, phosphorus, and potassium content of the plants was examined, using analytical

data for each of the five harvests between 11 and 44 days, by the method of multiple regression.

Where the purpose was to compare different sets of independent variables (e.g. analyses on different occasions) as bases for estimation of response, it would clearly be inappropriate to use different ranges of treatments. Accordingly, only those treatments were used in which a complete set of data for responses and for

TABLE 8

RESPONSES OF TOTAL NITROGEN CONTENT (PER CENT. OF DRY MATTER) AT 98 DAYS TO POTASSIUM SUB-TREATMENT, AS AFFECTED BY INITIAL NUTRIENT SUPPLY
Average Effects at Different Levels of Initial Treatments

N ₁	-0.09			K ₁	-0.99
N ₂	-0.26			K ₂	+0.02
N ₃	-0.20	P ₃	+0.18	K ₃	-0.05
N ₄	-0.30	P ₄	-0.52	K ₄	-0.13
N ₅	-0.19	P ₅	-0.29	K ₅	+0.11

Interactions between Initial Treatments

	N ₁	N ₂	N ₃	N ₄	N ₅
P ₃	+0.09	-0.31	+0.12	+0.55	+0.49
P ₄	-0.42	-0.28	-0.51	-0.53	-0.57
P ₅	+0.05	-0.20	-0.22	-0.92	-0.18
	N ₁	N ₂	N ₃	N ₄	N ₅
K ₁	-0.72	-0.80	-0.37	-1.49	-1.46
K ₂	+0.28	+0.03	-0.30	-0.12	+0.20
K ₃	+0.11	+0.39	-0.19	+0.25	-0.82
K ₄	+0.00	-0.52	-0.06	-0.28	+0.19
K ₅	-0.14	-0.41	-0.01	+0.13	+0.96
	K ₁	K ₂	K ₃	K ₄	K ₅
P ₃	-0.69	+0.71	+0.08	-0.11	+0.93
P ₄	-1.56	-0.39	-0.40	+0.01	-0.27
P ₅	-0.72	-0.26	+0.17	-0.31	-0.34

analyses on each of the occasions under comparison was available. For samples taken at 29, 37, and 44 days, these numbered 70, but if the earlier harvests were to be compared as well only 20 treatments could be included. This, of course, greatly reduced the precision with which regressions could be calculated, and accordingly both bases of computation (for 20 and for 70 treatments) were used for the later harvests.

In no case was the inclusion of analytical data for potassium found to increase the precision with which response to nitrogen could be estimated, over that obtainable

from the nitrogen and phosphorus content alone. Accordingly, potassium content has been ignored. On the other hand, where the multiple regression as a whole was significant, the contributions from the two other independent variables (nitrogen and phosphorus content) were both individually significant. In Tables 10–13 these two regression coefficients are quoted in mg/l per cent. change in nutrient content, together with their significance individually; the significance of the multiple regression as a whole is also presented in the form of a variance ratio.

TABLE 9
GROWTH RESPONSE TO NITROGEN (MG)

Nitrogen Levels	Potassium Levels	Phosphorus Levels				
		P ₁	P ₂	P ₃	P ₄	P ₅
N ₁	K ₁	+15	−557	+314	−505	+876*
	K ₂	−1	−151	+2130	+5171	+2844
	K ₃	−42	−254	+1515	+5933	+1357
	K ₅	+38	−233	−740	+2429	+3196
N ₂	K ₁	−1	+130	+1874*	+96	−362
	K ₂	−122	+30	+597	+2197	+1257
	K ₃	+122	−106	−554	−1485	+5389
	K ₅	+74	+35	+144	+3410	+2718*
N ₃	K ₁	+2	−104	+243	+131	−263
	K ₂	−12	−201	−84	+265*	−3199
	K ₃	−24	−102	−1198	−338	+29
	K ₅	−119	+199	+154	−5078	−587
N ₅	K ₁	+1	−5	+13	−209	+119
	K ₂	−75	+7	+114	+842	+2601*
	K ₃	+2	−7	+727*	−705	−1864
	K ₅	+28	+93	−145*	−3016	+885

* Fitted values, excluded from regression calculations.

(i) *Total Nitrogen*.—Where the total nitrogen content of the aerial part of the plant, together with its phosphorus content, was used to estimate the response (Table 10), it was found that the accuracy of estimation increased as the plant grew older. At 44 days, the regression reached very high significance and accounted for nearly a third of the total variation in response.

(ii) *Nitrate Nitrogen*.—Where data for nitrate nitrogen were used in place of total nitrogen content (Table 11) the significance of the regression was much less for analyses of the later harvests but reached a maximum in the intermediate harvests (29 and, perhaps, 22 days). At 29 days the response could be estimated almost as accurately from the nitrate content as it could from the total nitrogen content at 44 days.

(iii) *Organic Nitrogen*.—Soluble organic nitrogen contents (soluble nitrogen minus nitrate nitrogen) were more uniform than other nitrogen fractions, so it was not

TABLE 10
REGRESSION OF RESPONSE ON TOTAL NITROGEN AND PHOSPHORUS CONTENT

	Age (days)	b_N	b_P	F for Multiple Regression
Analyses for 20 treatments on each occasion	11	—835	1515	1.1
	22	—995*	4874*	3.9*
	29	—1214**	5180**	5.9*
	37	—783	4100	1.7
	44	—984**	6346**	6.5**
Analyses for 70 treatments on each occasion	29	—1023**	5366**	4.8*
	37	—1362***	7385***	8.0***
	44	—1471***	6523***	10.4***

* $P=0.01-0.05$.

** $P=0.001-0.01$.

*** $P<0.001$.

expected that they would provide a particularly accurate prediction of response. The results of the regression, shown in Table 12, confirm this. The regression is

TABLE 11
REGRESSION OF RESPONSE ON NITRATE NITROGEN AND PHOSPHORUS CONTENT

	Age (days)	b_N	b_P	F for Multiple Regression
Analyses for 20 treatments on each occasion	11	—3100	405	1.0
	22	—2175**	3546*	4.4*
	29	—2181*	4273*	3.7*
	37	—1562	3278	†
	44	—1667	4520*	2.8
Analyses for 70 treatments on each occasion	29	—3512***	4866***	8.0***
	37	—2431*	4363*	3.6*
	44	—2540*	3822*	4.0*

* $P=0.01-0.05$.

** $P=0.001-0.01$.

*** $P<0.001$.

† Regression mean square less than error mean square.

only significant at 44 days, and then only at the 5 per cent. level. In view of these results obtained with data for 70 treatments, the corresponding regressions at 11 and 22 days with more limited sets of data were not computed.

(iv) *Insoluble Nitrogen*.—Most of the nitrogen in all plants was in the insoluble fraction, so there was a close correlation between this fraction and total nitrogen content. Only one regression was calculated for this fraction—that for 44 days, at which harvest the regression for total nitrogen reached the highest level of significance.

TABLE 12

REGRESSION OF RESPONSE ON CONTENT OF NITROGEN FRACTIONS AND PHOSPHORUS: 70 TREATMENTS

Fraction	Age (days)	b_N	b_P	F for Multiple Regression
Soluble organic nitrogen	29	—956	1499	1.2
	37	—1904	1953	2.6
	44	—2417*	2046	4.1*
Insoluble nitrogen	44	—1907***	6523***	7.5***

* $P=0.01-0.05$.*** $P<0.001$.

The results (Table 12) show that the substitution of insoluble for total nitrogen reduced rather than increased the significance of the regression.

(v) *Choice of Organ*.—The only harvest at which a fair number of the plants were large enough to obtain separated organs for analysis was that at 44 days, but even

TABLE 13

REGRESSION OF RESPONSE ON NITROGEN AND PHOSPHORUS CONTENT OF DIFFERENT ORGANS AT 44 DAYS

Fraction	Organ	b_N	b_P	F for Multiple Regression
Total nitrogen (32 treatments)	Older leaves	—1259*	6050	2.6 .
	Younger leaves	—1296*	4349	2.2
	Whole tops	—1319*	5969	2.8
Nitrate nitrogen (27 treatments)	Midribs	—452	1800	†
	Whole tops	—1219	5212	1.7

* $P=0.01-0.05$.

† Regression mean square less than error mean square.

here most of the plants were too small for subdivision. The data available for comparison between organs are thus both rather limited in number and confined to larger plants, presumably with a more favourable nutritional status. Only two sample analyses will therefore be given. These were selected as being most likely to use the differences in nutrient content to the best advantage. For the comparison of older and younger leaves with the entire tops the total nitrogen content was

used, but for the comparison of midribs with the tops the nitrate content was used, because this varied over a particularly wide range (0–20,000 p.p.m.).

The results of these analyses, shown in Table 13, suggest that, on the basis of these data, no preference between older and younger leaves, or between either organ and the whole tops, can be made; and that the nitrate content of the midribs is not closely correlated with response.

IV. DISCUSSION

Among the treatment effects observed on nitrogen content, the general increase with increased nitrogen supply was to be expected; in fact, an increase in the proportion of a nutrient in plant dry matter with increased supply is usual and any exception calls for explanation. At higher levels of supply, though, the mean effects on nitrogen content are small. In accordance with general experience, the proportion of nitrate nitrogen is reduced at low levels of nitrogen supply—a greater proportion of the nitrogen taken up has been used promptly for protein synthesis. As for the effect of other nutrients, general experience is that, where increased supply of a nutrient increases growth, it results also in a decreased percentage content of other nutrients. This is true for the effects of potassium on nitrogen content in the present experiment—the increase from K_1 to K_2 effective in promoting growth is also effective in reducing nitrogen content. Such results are usually expressed in terms of “luxury consumption”—where potassium supply is limiting growth (K_1), nitrogen is taken up in excess of that which can be effectively used in growth, and additional potassium, by increasing growth, “dilutes” the nitrogen present. As usual with luxury consumption of nitrogen, the proportion of nitrogen in the form of nitrate increases. This effect of potassium supply on nitrogen content only shows itself where potassium is effectively limiting; it does not occur among the treatments with lower levels of phosphorus supply.

The effect of phosphorus supply on nitrogen content is very different, leading as it does to substantial increases. Since it was concurrently stimulating growth very markedly, nitrogen uptake was promoted in even greater proportion. Phosphorus is, of course, known to be involved in protein synthesis; but other factors are involved, for one finds the nitrate content increased by phosphorus supply even more than that of the other nitrogen fractions—i.e. the effects of phosphorus on nitrate uptake were even more marked than those on its reduction and utilization in protein synthesis. These effects of phosphorus on nitrogen content are more marked when nitrogen is in fully adequate supply—in which circumstances, of course, the phosphorus supply was able to exert its full influence on growth.

The wider range of variation in nitrate nitrogen than in total nitrogen content which is seen in these data has been very commonly observed. It rests on the fact that protein synthesis and the antecedent processes tend rapidly to use up all available nitrate when this is in short supply, but that when it is abundant it tends to be accumulated to some extent in the plant as a form of reserve. In accordance with this general rule, nitrate content also responds more markedly to the various nutritional treatments, whether they affect nitrogen uptake directly or indirectly.

In this experiment the effects of treatments on nitrate and total nitrogen content are remarkably similar, differing only in their magnitude.

The additional supplies of nutrients at 46 days had effects generally in line with those of the initial applications. The additional supply of nitrate generally increased the nitrogen content of the plants at 98 days; but where the plants already had a high nitrogen content (for instance with high initial nitrogen supply) this increase was small or negligible. Growth responses were also observed (Goodall, Grant Lipp, and Slater 1955) but clearly the nitrate uptake was stimulated more than the growth. Once again there is evidence that phosphorus favoured nitrogen uptake, for the plants which had received a high initial supply of phosphorus but little nitrogen showed a particularly marked increase in nitrogen content when supplementary nitrogen was supplied. Where potassium supply was limiting, the supplementary nitrogen led to increases in nitrogen content, presumably through luxury consumption.

The effects of supplementary supplies of phosphorus and potassium at 46 days are in general agreement with the effects of supplies of these elements at the outset. Extra phosphorus at 46 days increased the nitrogen content in phosphorus-deficient plants and in plants well supplied with nitrogen, in accordance with the marked interaction between the initial treatments with nitrogen and phosphorus. In potassium-deficient plants, potassium supply at 46 days reduced nitrogen content, especially where a substantial growth response was assured by adequate supplies of phosphorus and nitrogen.

Some of the results of the regression analysis are quite clear-cut. One of these is the progressive increase in significance of the regression of response on total nitrogen content up to the time of the supplementary application. This may be compared with the results of Crowther (1941), who found that the variation in different seasons in the yield of cotton in the Sudan was closely related to the nitrogen content of the seedlings within 2 weeks of sowing; the close correlation continued through the next month, but then decreased steadily. In comparing these results, however, it should be remembered that Crowther's differences in yield were mainly due to differences in accumulated nitrogen reserves and nitrification activity in the soil, and thus could be related only indirectly to the prospective responses to nitrogenous top-dressings applied during growth.

The high correlation of nitrogen response with nitrogen content occasions no surprise, but the importance of phosphorus content was unexpected. *Prima facie*, one would imagine that the *slope* of the nitrogen response curve might reflect the current nitrogen status only, the status in respect of other nutrients affecting the rate of change in slope rather than its initial value (the second rather than the first differential). This assumption cannot definitely be denied from the present data, for the supplementary nitrogen supply was far from infinitesimal; but it is made to seem unlikely. In this, the present work confirms the results of Lundegårdh (1941, 1943, 1951) who, it will be recalled, found that the response of oat plants in different localities to initial nitrogen supply was related to both nitrogen and phosphorus content of the control plants at flowering.

In contrast to phosphorus, potassium content seems irrelevant to the forecasting of response to nitrogen. This would not necessarily imply that potassium status has no influence on response to nitrogen (though this appears to have been the case in the present experiment—see Goodall, Grant Lipp, and Slater 1955); if potassium deficiency had been associated with a reduced response to nitrogen, as is often the case, this would have been reflected in an increased nitrogen content, enabling one to forecast the change in response potentialities without recourse to potassium analyses. Lundegårdh does not refer to any influence of potassium on the relationship between nitrogen content and nitrogen response; presumably it was missing from his results likewise.

TABLE 14

REGRESSION OF RESPONSE ON NITROGEN AND PHOSPHORUS CONTENT AT 44 DAYS: ANALYSIS OF VARIANCE

	Degrees of Freedom	Mean Square	<i>F</i>	<i>P</i>
Linear regression*	2	20831764	10.62	<0.001
Quadratic terms†	3	6216438	3.17	0.01–0.05
Error	67	1961659		

$$*y = 2254 - 1280x_1 + 5866x_2.$$

$$†y = 4557 - 3981x_1 + 17292x_2 + 636x_1^2 - 3593x_2^2 - 3374x_1x_2.$$

Lundegårdh showed the relationship between nitrogen content and nitrogen response, at all phosphorus levels except the lowest, as curvilinear; but he applied no tests to the deviations from linearity. That has been done for the present data. In the case of the analyses for total nitrogen and phosphorus of samples collected at 44 days (when the relationship was closest), an expression of the following form was fitted:

$$y = a + bx_1 + cx_2 + dx_1^2 + ex_2^2 + fx_1x_2,$$

where y is the response to supplementary nitrogen in milligrams, while x_1 and x_2 are the content of nitrogen and phosphorus (per cent. of dry matter) at 44 days. The results, as shown in Table 14, support Lundegårdh's view, though the improvement over the simple linear expression only just reaches significance.

The analyses for different nitrogen fractions do not suggest that any of them provides a better basis for forecasting response than the total nitrogen content, except that nitrate analyses may enable a satisfactory forecast to be given at an earlier date. At 29 days, nitrate analyses provided forecasts almost as good as those based on total nitrogen content at 44 days; and already at 22 days from sowing some information on the prospective response was available.

The analyses of different organs were unfortunately too few to enable a satisfactory test of their relative value as diagnostic material to be made. It would certainly seem that the difference in this respect between older and younger leaves

was nothing like as great for nitrogen deficiency in lettuce as for potassium deficiency in barley (Goodall 1948). The failure of nitrate content of midribs to show a relation-

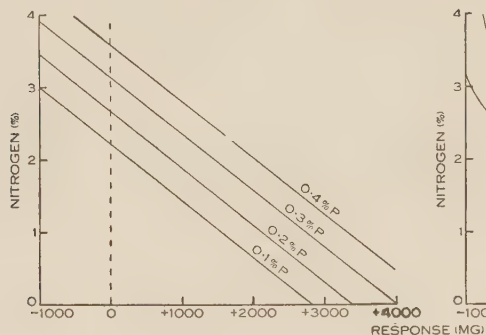


Fig. 11

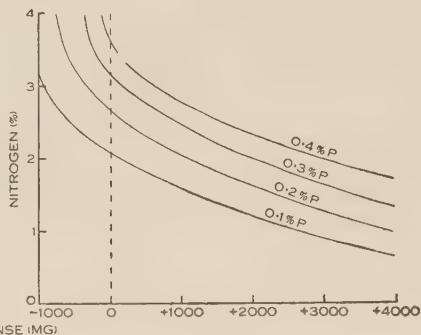


Fig. 12

Figs. 11 and 12.—Relation between response to nitrogen and total nitrogen content at different phosphorus levels; analytical data for 44 days. Linear regression (Fig. 11); quadratic regression (Fig. 12).

ship with response to nitrogen is noteworthy and it is at variance with many recorded results (Carolus 1938; Harrington 1944; Ulrich 1942, 1946, 1948, 1950). It has often

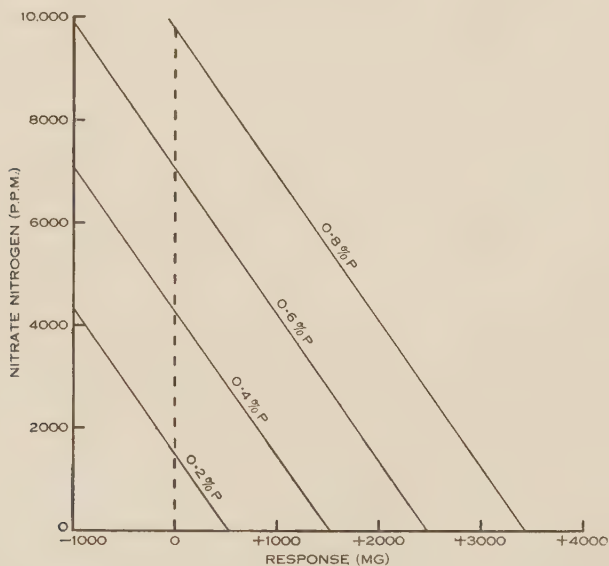


Fig. 13.—Relation between response to nitrogen and nitrate nitrogen content at different phosphorus levels; analytical data for 29 days.

been claimed (e.g. Emmert 1935) that this is a good index of current uptake by the roots and hence of the supplies of nitrogen available for synthesis and growth; if this were so, a close relationship with response would certainly be expected. It is

possible that difference in current uptake of nitrate may have been masked to some extent by changes in the rate of nitrate reduction in the roots; the relative importance of different sites of nitrate reduction seems never to have been investigated in the lettuce.

To pass to practical conclusions regarding the nutrition of lettuce in the field would be beyond the scope of this paper, and would not be justified by the data. It may, however, be of interest to note the most clear-cut results as presented in Figures 11–13. These show the response as related to nitrogen content (total nitrogen at 44 days and nitrate nitrogen at 29 days), at four arbitrarily chosen levels of phosphorus content. In respect of total nitrogen content, both linear (Fig. 11) and quadratic (Fig. 12) expressions were used. It will be seen that positive responses were to be expected only where the nitrogen content did not exceed the values given in Table 15.

TABLE 15
NITROGEN CONTENT CORRESPONDING TO ZERO RESPONSE

29 Days from Sowing		44 Days from Sowing		
Phosphorus (%)	Nitrate Nitrogen (p.p.m.)	Phosphorus (%)	Total Nitrogen* (%)	Total Nitrogen† (%)
0.2	1520	0.1	2.22	2.09
0.4	4290	0.2	2.68	2.65
0.6	7060	0.3	3.14	3.16
0.8	9830	0.4	3.59	3.54

* Linear expression.

† Quadratic expression.

It should be remembered that the samples for analysis were taken at fixed *times*, and not at corresponding stages of morphological or physiological development in the different treatments. With such a wide range of treatments, it would in fact have been almost impossible to find stages which could be said to correspond. Given a narrower range of conditions, sampling on the basis of developmental stage might be possible, and it would certainly be more practicable for advisory work under field conditions. It might then well be found that the relation of response to analytical data was closer for samples taken on this basis than samples at a fixed time.

V. ACKNOWLEDGMENTS

Once again, we have the pleasure and duty of expressing our thanks to our colleagues at Melbourne University for their help, and to Professor J. S. Turner, in whose department and with whose encouragement the work was carried out. Some of the computations were performed and diagrams drawn by Miss Julie Bromley, University of Reading. The work was supported by the Research Grant of the University of Melbourne.

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COMPOSITION OF THE NITROGEN FRACTION OF APPLE TRACHEAL SAP

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[Manuscript received February 20, 1957]

Summary

Organic nitrogenous compounds accounted for most of the nitrogen present in apple tracheal sap. Aspartic acid, asparagine, and glutamine were quantitatively the most important compounds. Glutamic acid and other amino acids were also present as well as a peptide-like substance. While apple variety, rootstock, or manurial treatment may have had effects on level of nitrogen in tracheal sap they seem to have had little effect on proportions of nitrogenous compounds present. Through the growing season, however, there was a definite change in proportions of some of the constituents. The composition of the soluble nitrogen fraction of leaves and fruits showed distinct differences from the composition of tracheal sap.

I. INTRODUCTION

Seasonal fluctuation in levels of nitrogen in tracheal sap extracted from the xylem of apple trees has already been described (Bollard 1953*b*). In the present paper a description of the nature of the nitrogenous compounds in this sap is given, and an account of some of the factors influencing their amount. The relation of these compounds to the soluble nitrogen fraction of leaves and fruit is also considered. A brief report of some of these results has already been given (Bollard 1953*a*).

II. MATERIAL AND METHODS

(a) *Extraction of Tracheal Sap*

Shoots consisting of only previous and current season's growth cut from mature apple trees were used for most sap extractions and all shoots were collected between 8 and 9 a.m. Each sap sample was extracted from shoots of a single tree by the methods previously described (Bollard 1953*b*). Early in the season, when total nitrogen content was high, about 10 ml of sap was enough for all determinations; later in the season, when total nitrogen had fallen, about 25 ml of sap was necessary.

(b) *Estimation of Nitrogen Fractions*

(i) *Total Nitrogen*.—Kjeldahl determinations were made on aliquots of usually 2 ml sap. No nitrate has been detected qualitatively in the sap and so no precautions were taken to include nitrate in the total nitrogen.

(ii) *Ammonia Nitrogen*.—Sap aliquots (2, 3, or 5 ml) were distilled *in vacuo* at 40°C with phosphate-borate buffer (Pucher, Vickery, and Leavenworth 1935). Ammonia was absorbed in dilute sulphuric acid and determined after redistillation from the Markham apparatus (Markham 1942).

(iii) *Glutamine-amide Nitrogen*.—Sap aliquots were subjected to mild hydrolysis (Vickery *et al.* 1935) by boiling with phosphate buffer at pH 6.5. Ammonia liberated

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was determined as above. Any increase in ammonia was due to glutamine breakdown and the nitrogen of this ammonia increment was designated glutamine-amide nitrogen.

(iv) *Asparagine-amide Nitrogen*.—Sap aliquots were hydrolysed in N sulphuric acid (Pucher *et al.* 1935). Ammonia liberated was determined as above. Any increase in ammonia nitrogen over that due to free ammonia and glutamine-amide was designated asparagine-amide nitrogen.

(v) *Amino Nitrogen*.—Amino nitrogen was determined in the Van Slyke manometric apparatus using nitrous acid (Milton and Waters 1949). Sap from which the ammonia had been previously removed was acidified before use. The value obtained was corrected for the presence of glutamine by subtracting 80 per cent. of the glutamine-amide nitrogen value (Vickery *et al.* 1935).

(vi) *Amide + Amino Nitrogen*.—This term is used for the sum of ammonia nitrogen, glutamine-amide nitrogen, asparagine-amide nitrogen, and amino nitrogen.

(vii) *Nitrate*.—The presence of nitrate in sap samples was tested for qualitatively after reduction to nitrite using sulphanilic acid and α -naphthylamine (Feigl 1947).

(c) *Chromatography of Sap Samples*

Aliquots of tracheal sap were frozen and concentrated *in vacuo* over sulphuric acid. This material was then ready for application to the chromatography paper. In general, enough concentrated sap to contain 10 μg nitrogen was used for one-way chromatograms and enough to contain 15–30 μg for two-way runs. Chromatography was also carried out on samples of sap which had been hydrolysed with 6N hydrochloric acid for 18 hr.

Chromatograms were run on Whatman No. 1 filter paper, using as solvents 80 per cent. phenol in water and butanol–acetic acid–water (5 : 1 : 4 v/v). Following chromatography, amino acid spots were revealed by spraying with ninhydrin (0.1 per cent. in isopropanol). After drying, the sheets of paper were heated in an oven at 80–85°C for 5 min.

Quantitative determinations of amino acids in sap samples were made using the method of Wellington (1952, 1953).

(d) *Chromatography of Leaf and Fruit Samples*

Samples of about 2 g of freshly collected leaf (or 6 g of fruit) were extracted for 3 min in six successive 25-ml portions of 87 per cent. ethanol in a "Nelco" homogenizer at room temperatures. All portions of alcohol were pooled and passed through a column of "Zeo-Karb 215" which had previously been washed with 87 per cent. ethanol. Amino acids were eluted from the column with 0.15N ammonia. Using this procedure it was possible to obtain the amino acids of leaf or fruit in solution free of sugar, colouring matter, etc. This eluate was reduced in volume at a temperature below 40°C and aliquots calculated to contain 30 μg nitrogen before application to the column (i.e. while chlorophyll etc. was still present) were chromatographed using the methods already described.

III. NATURE OF NITROGENOUS COMPOUNDS PRESENT IN TRACHEAL SAP OF APPLE TREES

(a) *Nitrogen Balance Sheets*

Determinations of nitrogen present in ammonia, glutamine-amide, asparagine-amide, and amino forms as well as of total nitrogen were made on a series of apple tracheal saps collected at different times from several varieties on several root-stocks. Results are given in Table 1. It can be seen that the differences between amide plus amino nitrogen and total nitrogen are small and probably attributable to experimental error. No nitrate could be detected in the sap. Thus, over a considerable part of the growing season, the nitrogen in the tracheal sap of apple trees of a number of varieties on several stocks is present very largely as organic nitrogenous compounds. Asparagine and glutamine usually account for over 50 per cent. of the total nitrogen.

(b) *Amino Acids Present in Tracheal Sap*

The foregoing work showed that asparagine and glutamine were important compounds in apple tracheal sap and also indicated that other amino compounds were present. The nature of the unknown compounds has been elucidated using paper chromatography.

Chromatography of many sap samples from various varieties at different times and under different conditions (see Sections IV and V) has always revealed the presence of aspartic acid, asparagine, and glutamine in largest amounts. Lesser amounts of glutamic acid were present while also occurring were serine, threonine, arginine, methionine, valine, and leucine. Sometimes present in trace amounts were alanine, tyrosine, γ -aminobutyric acid, and phenylalanine. Quantitative measurements of most of these compounds are presented in Section IV.

After acid hydrolysis there were changes in amounts of some compounds. Apart from disappearance of the two amides, with corresponding increases in glutamic and aspartic acids, there was an increase in serine and alanine while glycine and, in lesser amounts, lysine, cystine, and, sometimes, cysteic acid appeared. These changes probably indicate that a peptide (or peptides) was originally present in the sap.

IV. VARIATION IN THE LEVEL AND COMPOSITION OF THE NITROGEN FRACTION OF TRACHEAL SAP

It was apparent in early work that changes occurred both in the level of nitrogen and nature of the nitrogenous compounds present in apple tracheal sap. Some of the factors likely to influence these were further investigated.

(a) *Variety*

No differences attributable to variety have been found in comparisons of the nitrogenous compounds present in the tracheal sap from trees of the following varieties: Delicious, Dougherty, Granny Smith, Gravenstein, Jonathan and Sturmer.

TABLE 1
AMOUNTS OF VARIOUS NITROGENOUS COMPOUNDS AND TOTAL NITROGEN IN TRACHEAL SAP FROM APPLE TREES
Results expressed as μg nitrogen/ml of sap

Sampling Date	Source of Sap		Ammonia Nitrogen	Glutamine-amide Nitrogen	Asparagine-amide Nitrogen	Amino Nitrogen	Total Ammonia, Amide, and Amino Nitrogen	Total Nitrogen (determined by Kjeldahl method)
	Variety	Stock						
23.x.54	Gravenstein	Northern Spy	1	11	21	88	121	121
1.xi.54	Delicious	Malling XII	4	22	30	105	161	158
1.xi.54	Delicious	Malling XVI	6	16	32	103	157	155
1.xi.54	Gravenstein	Northern Spy	2	8	23	71	104	98
11.xii.54	Delicious	Malling XII	1	10	7	36	54	54
11.xii.54	Delicious	Malling XVI	1	4	20	56	81	77
11.xii.54	Gravenstein	Northern Spy	1	2	12	12	27	20
24.iii.55	Granny Smith	Malling XVI	0	1	2	13	16	17
24.iii.55	Granny Smith	Northern Spy	0	2	2	13	17	16
24.iii.55	Granny Smith	Malling XII	0	1	2	13	16	19

(b) Time of Year

It has already been shown (Bollard 1953*b*) that there is a considerable regular seasonal variation in level of nitrogen in apple tracheal sap. A low level (10 $\mu\text{g/ml}$) during the dormant period was found to be followed by a sudden rise (to 150 $\mu\text{g/ml}$) at blossom time. This high level was maintained for about 3 weeks and there was then a gradual decline till, by harvest time, the dormant level was again reached.

TABLE 2

PROPORTIONS OF VARIOUS NITROGENOUS COMPOUNDS PRESENT IN APPLE TRACHEAL SAP FOR GRANNY SMITH TREES AT SEVERAL TIMES DURING THE GROWING SEASON

Results expressed as per cent. nitrogen contributed by each compound. + = present but less than 1 per cent.

Compound	Sampling Date					
	12.x.54	19.x.54	26.x.54	29.xi.54	29.xii.54	26.i.55
Aspartic acid	12	10	22	31	58	41
Asparagine	65	59	52	65	34	48
Glutamic acid	+	+	+	1	2	3
Glutamine	20	27	24	4	6	7
Serine	+	1	+	+	+	2
Threonine	2	1	1	+	+	+
Methionine + valine	1	1	1	+	+	1
Leucine	1	+	+	+	+	+
Total nitrogen ($\mu\text{g/ml}$)	41	117	131	48	22	12
Percentage of total nitrogen contributed by aspartic acid + asparagine	77	69	74	96	92	89

Chromatography of a series of sap samples showed that the same nitrogenous compounds were generally present through the year. Aspartic acid and asparagine were the most important constituents quantitatively at all times. Glutamine tended to decrease relatively as the season progressed while glutamic acid tended to increase. Arginine showed a striking increase towards the end of the season. Serine, threonine, methionine or valine, and leucine were relatively abundant at blossom time, when nitrogen levels were high, but soon afterwards were scarcely detectable. The peptide-like substance was apparently present at all times as the same amino acids appeared on hydrolysis of each sap sample.

Proportions of individual compounds in a series of sap samples from Granny Smith trees were determined using the quantitative paper chromatographic method of Wellington (1952, 1953). Results are given in Table 2. These results show the same sort of relationships as those already indicated by the qualitative chroma-

tography. Asparagine and aspartic acid were clearly the most important compounds contributing from 69 to 96 per cent. of the total nitrogen.

(c) *Rootstock and Fertilizer Treatment*

Comparison of nitrogen contents of sap samples taken from trees on different rootstocks in two experimental blocks (Woodhead, McKenzie, and Farmer 1954; Woodhead and McKenzie 1955) and from trees receiving different fertilizer treatments (Tiller, Roberts, and Bollard, unpublished data) showed some significant differences at particular times especially in samples taken when nitrogen levels were high. More detailed data would be necessary to establish any overall differences attributable to rootstock or fertilizer treatment.

No differences in nitrogenous compounds present in the sap from trees on different rootstocks or receiving different fertilizer treatments could be detected. Examination of saps from a range of unworked stocks also revealed no differences.

V. RELATION OF TRACHEAL SAP CONSTITUENTS TO COMPOSITION OF SOLUBLE NITROGEN FRACTION OF LEAF AND FRUIT

It is evident that a substantial portion, at least, of the nitrogen reaching the shoot must travel from the root in the tracheal sap. Though McKee and Urbach (1953) have already given a full account of the amino acids present in fruit and leaves of the Granny Smith apple, it was considered of interest to compare the soluble nitrogenous substances present in leaves and fruit with the nitrogen compounds concurrently present in tracheal sap.

Tracheal sap, leaf, and fruit samples were collected on two occasions from the same group of Granny Smith trees on Malling XII stock as follows:

Sampling Date	Nitrogen Content of Tracheal Sap ($\mu\text{g N/ml}$)	Average Weight of Fruit (g)
Nov. 22	57	2.9
Jan. 12	24	56.0

Examinations were made of two-way chromatograms run using tracheal sap and alcoholic extracts from leaf and fruit.

The first samples were taken at a time when there were comparatively large amounts of glutamine and asparagine in the sap in addition to aspartic acid, and other amino acids were present to only a slight extent. The amino acids in the leaf were present in very different proportions from those found in the sap. In the leaves, alanine and glutamic acid were most important quantitatively, with aspartic acid and serine next most abundant. The two amides were not prominent. In the fruit, asparagine was unquestionably the most abundant amino acid while only a trace of glutamine was present. Alanine, serine, glutamic acid, and aspartic acid were also prominent.

The second samples were taken at a time when aspartic acid had become more important in the tracheal sap. In the leaf during the same period, this compound was

not so important quantitatively as alanine, serine, or glutamic acid. In the fruit, asparagine was again the most important compound with alanine and serine also prominent.

These results with leaf and fruit extracts are in close agreement with those recorded by McKee and Urbach (1953) but it is interesting to note that glycine was present in the leaf and fruit and cysteic acid in the leaf at both sampling times. These compounds were not reported by McKee and Urbach nor did they occur free in the tracheal sap.

VI. DISCUSSION

It would seem that the nitrogen in tracheal sap of apple trees is mostly present in organic compounds. Ammonia nitrogen was present in small proportion only and nitrate nitrogen has not been detected. These results are in accordance with those of Thomas (1927) who showed that nitrate could be detected in only the thinnest roots of apple trees, and with those of many workers who have shown that nitrates are not normally present in apple foliage. With heavy applications of nitrate Stuart (1932) found some in apple leaves but its presence was usually associated with damage. This is of interest in view of the fact that nitrate apparently occurs normally in all parts of many plants.

Work with radioactive tracers (Stout and Hoagland 1939) has confirmed the older view that upward translocation of, at least, some elements takes place in the xylem. The movement of nitrogen in the xylem has not been confirmed in this way but the present work has shown that nitrogen occurs abundantly in tracheal sap and a substantial portion, at least, of the nitrogen reaching the shoots must come through this channel. The occurrence of organic nitrogenous compounds in a sap from the xylem is perhaps surprising as the older view was that the transpiration stream simply carried upwards absorbed but unassimilated inorganic nutrients.

It is clear that, in the apple tree, stages in the assimilation of nitrogen are separated morphologically. Normally, incorporation of inorganic nitrogen into organic compounds would seem to be largely accomplished in the roots. The root system then supplies upper parts of the tree with a defined range of amino acids, amides, and peptide-like substances. Further metabolism of nitrogen in leaves and fruit can then occur using the compounds supplied in tracheal sap as starting materials.

It is not certain that nitrogenous compounds present in tracheal sap are the immediate products of incorporation of inorganic nitrogen. The root of an apple tree is a bulky perennial organ and there is ample opportunity for storage of nitrogen and some or all of the nitrogen present in tracheal sap may well result from hydrolysis of storage proteins. There is some evidence (Steward and Thompson 1954) that asparagine may be closely associated with protein breakdown. There is no doubt that, in apple tracheal sap, aspartic acid and asparagine are quantitatively more important than glutamic acid and glutamine and this may indicate that protein breakdown occurs in roots. It is not impossible that all nitrogen absorbed and assimilated by apple roots passes through a transitory storage phase.

The pattern of amino acids etc. present was remarkably constant through a range of varieties, stocks, and manurial treatments. The presence of amino acids such as serine, threonine, etc. raises the question of whether these compounds are necessarily synthesized in the root or whether the leaf is also capable of their synthesis. The peptide-like substance is constantly present but there is no indication as to what role, if any, it plays in further nitrogen metabolism. There is some evidence that nitrate cannot be metabolized by apple leaves but there is little other indication of how specific are the needs of upper parts of the plant. Changes in the proportions of different amino acids present in tracheal sap occurred through the growing season. It is not possible to say if these are simply changes consequent on changes in root growth or metabolism and of little importance to the shoot.

Comparison of tracheal sap constituents with compounds present in soluble nitrogen of leaves and fruit has produced interesting differences, which are difficult to explain in the present state of our knowledge. While it is reasonable to believe that the leaves receive a significant proportion of their nitrogen from tracheal sap one cannot be sure about fruits. It may be that tracheal sap supplies some nitrogen directly to developing fruits but it is also possible that some of the fruit nitrogen arrives after passage through, and possible transformation in, leaves.

There have been suggestions recently (Steward and Thompson 1954) that, despite the analogous nature of glutamine and asparagine, these two compounds are physiologically dissimilar. The present work has shown that, in apple, these two compounds seem to have different roles. While both were present in tracheal sap asparagine was more important quantitatively as a translocatory substance at all times, and particularly after the initial high nitrogen levels had dropped. In leaves, both amides were present in comparatively low concentration, glutamic acid and other amino acids being present in greater quantity. Trees more adequately supplied with nitrogen might have shown higher concentrations of one or both amides, or it may be that it is a result of translocation of these compounds away from leaves. In this connection it is interesting to note that in leaves soluble nitrogen is usually only 3-5 per cent. of total nitrogen while in fruit it is usually about 50 per cent. In fruits only a trace of glutamine was present and asparagine was the predominant compound.

VII. ACKNOWLEDGMENT

The assistance of N. A. Turner of this Research Station in much of the work reported here is acknowledged.

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NITROGENOUS COMPOUNDS IN TRACHEAL SAP OF WOODY MEMBERS OF THE FAMILY ROSACEAE

By E. G. BOLLARD*

[*Manuscript received February 20, 1957*]

Summary

Nitrogen present in the tracheal sap of a range of woody species of the family Rosaceae was completely accounted for by a number of organic compounds, notably aspartic acid, asparagine, and glutamine. A striking similarity was shown to the composition of apple tracheal sap.

I. INTRODUCTION

Until recently it was generally believed that nitrogen moved through the xylem of plants in an inorganic form, but it has been shown (Bollard 1957) that, throughout the year, aspartic acid, asparagine, and glutamine are the most important nitrogenous compounds in the tracheal sap of apple trees. The remainder of the nitrogen fraction consists of a number of other amino acids and a peptide-like substance. The question remains as to whether this phenomenon is peculiar to apple trees or occurs more widely. In this paper an account is given of an examination of the nitrogenous compounds in the xylem sap of other woody species (mainly fruit species) of the family Rosaceae.

II. MATERIALS AND METHODS

Shoots were collected from trees growing in gardens or commercial orchards in the Auckland district. Sap extraction was carried out as previously described (Bollard 1953).

Methods for estimation of total nitrogen, ammonia nitrogen, glutamine-amide nitrogen, asparagine-amide nitrogen, and amino nitrogen and chromatographic methods have been previously described (Bollard 1957). Amino acids were quantitatively determined by the chromatographic method of Wellington (1952, 1953).

III. RESULTS

(a) *Nitrogen Balance Sheets*

In Table 1 are given the results of analyses for the several nitrogen fractions in a series of sap samples from trees of rosaceous fruit species, representative results with apple tracheal sap being included for comparison. The first sample from each species was collected at full blossom, when, by analogy with apples, the nitrogen level in the sap might be expected to be at its maximum. The second sample was taken at least 4 weeks later when, in apples, the nitrogen level would have dropped considerably.

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TABLE 1
AMOUNTS OF VARIOUS NITROGENOUS COMPOUNDS IN TRACHEAL SAP FROM TREES OF THE FAMILY ROSACEAE
Results expressed as mg N/ml sap

Tree	Variety	Sampling Date	Ammonia Nitrogen	Glutamine-amide Nitrogen	Asparagine-amide Nitrogen	Amino Nitrogen	Total Ammonia, Amide, and Amino Nitrogen	Total Nitrogen (determined by Kjeldahl method)
Pear	Beurre Bosc.	24.x.54	1	11	27	80	119	124
		28.xi.54	1	2	8	27	38	38
Quince	Missouri Mammoth	29.ix.54	0	7	6	22	35	34
		13.xi.54	0	2	2	12	16	12
Japanese plum	Sharp's Early	29.ix.54	1	24	57	142	224	221
		18.xi.54	1	4	13	41	59	50
Myrobalan plum		29.ix.54	1	8	34	77	120	128
		18.xi.54	0	3	7	24	34	27
Peach	Golden Queen	29.ix.54	2	93	103	308	506	511
		17.xi.54	1	6	14	38	59	59
Apricot	Oullin's Early	30.ix.54	1	124	70	317	512	484
		11.xii.54	1	4	10	36	51	51
Flowering cherry		30.ix.54	1	40	18	168	227	275
		17.xi.54	3	4	8	55	70	77
Apple	Gravenstein	23.x.54	1	11	21	88	121	121
		11.xii.54	1	2	12	12	27	20

It can be seen that the nitrogen levels in sap from pear, quince, and Myrobalan plum were similar to those in sap from apples. Other stone fruits, however, especially peach and apricot, had more nitrogen in the sap at blossom time. With the possible exception of the first sampling from flowering cherry, the differences between total nitrogen estimated separately and the sum of ammonia, amide, and amino nitrogen were small and probably within the limits of experimental error. It is thus apparent that the nitrogen in the tracheal sap of these trees consisted largely of these organic nitrogenous compounds. When the amino nitrogen of asparagine and glutamine is

TABLE 2

PROPORTIONS OF VARIOUS NITROGENOUS COMPOUNDS PRESENT IN TRACHEAL SAP FROM VARIOUS FRUIT TREES

Results expressed as per cent. nitrogen contributed by each compound. + = present but less than 1 per cent.

Compound	Source of Sap and Sampling Date			
	Pear 22.x.54	Quince 22.xii.54	Myrobalan Plum 24.ix.54	Apricot 20.xii.54
Aspartic acid	1	43	18	24
Asparagine	56	51	47	64
Glutamic acid	+	1	+	1
Glutamine	40	4	34	10
Serine	1	+	+	+
Threonine	1	+	+	+
Methionine + valine	1	+	1	+
Leucine	+	+	+	+
Alanine	+	+	+	+
Total nitrogen ($\mu\text{g/ml}$)	224	35	239	122
Percentage of total nitrogen contributed by aspartic acid + asparagine	57	94	65	88

taken into account these two compounds together usually accounted for over 50 per cent. of the total nitrogen as with apples. With apricot and flowering cherry at the first sampling there was more glutamine than asparagine but with these species at the second sampling and with all others at both samplings asparagine was predominant.

(b) Amino Acids Present in Tracheal Sap

A chromatographic examination of the individual nitrogenous constituents of these sap samples has also been carried out. The pattern of amino acids revealed was strikingly similar to that shown by apple tracheal sap. Aspartic acid, asparagine, and glutamine were present in largest amounts in all cases. Glutamic acid was

present in lesser amounts while also evident were traces of serine, threonine, methionine or valine, leucine, and arginine. Sometimes present in traces were alanine, γ -aminobutyric acid, and glycine. One noticeable difference from apple sap was shown by peach and cherry sap both of which contained small amounts of proline.

The tracheal sap of two other members of the Rosaceae, *Rubus fruticosus* L. and *Spiraea thunbergii* Sieb., revealed a similar range of nitrogenous compounds.

A further similarity to apple sap was revealed by chromatography of acid-hydrolysed sap samples which showed increases in serine and alanine, and usually the appearance of some glycine, lysine, cystine, and cysteic acid. It is apparent that in these saps also there is present a peptide-like substance or substances.

(c) Quantitative Relations

Determinations of the proportions of individual amino acids in some sap samples were made using quantitative chromatography. Some typical results are given in Table 2. In all cases asparagine was quantitatively the most important constituent. It was clear that in sap samples collected early in the season when nitrogen was high (e.g. pear and Myrobalan plum) glutamine was quantitatively more important than aspartic acid. In later samples (e.g. quince and apricot) when nitrogen levels were lower this position had been reversed, just as it is with apples over the same period. Even early in the season, however, aspartic acid plus asparagine in all species accounted for over 50 per cent. of the nitrogen present while this proportion became much higher later.

IV. DISCUSSION

It is clear that the presence of organic nitrogenous compounds in tracheal sap is not peculiar to apples. Nitrogen in the tracheal sap of a range of woody species of the Rosaceae was made up largely of the same range of amino acids and amides as had been demonstrated for apple sap. In seasonal change of composition too there was a striking similarity to apple sap. One outstanding difference was the presence of proline in sap from two species of stone fruit. The presence of similar peptide-like substances in the saps of all species examined gives added interest to the occurrence of such compounds in apple tracheal sap.

V. ACKNOWLEDGMENT

The assistance of N. A. Turner of this Research Station in much of the work reported here is acknowledged.

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TRANSLOCATION OF ORGANIC NITROGEN IN THE XYLEM

By E. G. BOLLARD*

[*Manuscript received February 20, 1957*]

Summary

Determinations have been made of the nitrogenous compounds present in xylem sap from a range of dicotyledons, monocotyledons, and gymnosperms.

Nitrate nitrogen was present in sap from a few species in appreciable amounts but in most was either absent or present as a trace only.

All saps contained a range of amino acids and amides. Glutamine and asparagine were quantitatively the most important compounds present in nearly all species. Some saps contained either citrulline or allantoic acid as a major constituent.

I. INTRODUCTION

The demonstration (Bollard 1957*b*) that nitrogen present in the xylem sap of woody members of the family Rosaceae is largely made up of a specific group of amides and amino acids raises the question of transport of nitrogen through the xylem of plants in general. While nitrate is known to be a normal constituent of the foliage of many plants, and is presumably transported there as such, it is absent from leaves of several members of the Rosaceae. Knowledge of nitrogenous compounds supplied to the shoot by the root system is basic to study of further nitrogen metabolism in plants. Extension of the technique for extraction of xylem sap (Bollard 1953*a*) to a wide range of woody and herbaceous plants has enabled an examination to be made of the forms of nitrogen present in xylem sap from a wide range of plants. A brief report (Bollard 1956) of some of these results has already been given.

II. METHODS

(a) Sap Extraction

The sap-extraction technique previously used on apples (Bollard 1953*a*) has been successfully applied to a number of herbaceous and woody species. With woody dicotyledons little difficulty was encountered, sap being successfully extracted from many plants, though the quantities obtained varied from species to species. Species with vessels of wide aperture, such as climbers, yielded large quantities of sap (e.g. 20 ml from a 1-m long shoot). Four species only yielded sap in quantities too small for most purposes. Some plants have stems with no secondary wood development but which are yet rigid enough to withstand the application of vacuum.

Monocotyledonous species which produce woody stems presented no difficulty and sap could also be extracted from those species with sufficiently robust flower stalks, but most monocotyledonous plants have a growth habit which precludes the use of this technique.

Despite the difference in structure of the xylem-conducting element in gymnosperms, sap was readily extracted from woody shoots of these plants.

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The original users of this extraction technique (Bennett, Anderssen, and Milad 1927) referred to the product obtained as tracheal sap. The wider applicability of the method has now rendered this name inappropriate and future publications from this Laboratory will refer to the liquid obtained as xylem sap.

(b) *Extraction of Leaf Nitrate*

Leaves were extracted in Morgan's solution (Nicholas 1948).

(c) *Detection of Nitrate*

Nitrate in the leaf extracts or sap samples was detected, after reduction to nitrite, using sulphanilic acid and α -naphthylamine (Feigl 1947).

(d) *Paper Chromatography*

Chromatographic separation and detection of amino acids in sap samples were carried out as previously described (Bollard 1957a). Aliquots of sap calculated to contain 15 μ g of nitrogen were used for each chromatogram. No quantitative measurements of individual compounds were made but each substance apparent after separation on a chromatogram was given an approximate assessment into one of three categories based on the following:

Trace constituent—when containing no more than 1 μ g of nitrogen.

Present in some quantity—when containing between 1 and 5 μ g of nitrogen.

Major constituent—when containing more than 5 μ g of nitrogen.

Allantoin, allantoic acid, citrulline, and urea were detected on paper chromatograms by spraying with a 2 per cent. solution of *p*-dimethylaminobenzaldehyde in 95 per cent. ethanol, acidified to normality with hydrochloric acid. These four compounds give a bright yellow colour with this reagent, citrulline reacting quickly and the others appearing on drying in a warm stream of air. This reagent can also be used on chromatograms which have been sprayed with ninhydrin; in these circumstances, citrulline, which has already reacted with ninhydrin, does not produce a yellow colour. The R_F values of allantoin and allantoic acid on chromatograms were:

	Phenol	Butanol-Acetic Acid
Allantoin	0.50	0.28
Allantoic acid	0.20	0.15

III. RESULTS

Measurements were made on a single member from each of 116 dicotyledonous families. Results from 31 species are given in Table 1. These were selected on the basis of botanical orders, i.e. one species of each of the 31 orders represented in the range of plants tested. Results from the examination of six monocotyledonous plants and nine gymnosperms are given in Tables 2 and 3.

Of the dicotyledons, 44 species had no nitrate extracted from the leaves while 60 had a trace only. Four species contained nitrite as well as nitrate. In 68 of the 110

TABLE 1 (Continued)

[illegible]

TABLE 3

LEAF NITRATE, XYLEM SAP NITROGEN CONTENT, AND COMPOUNDS PRESENT IN XYLEM SAP FROM A RANGE OF GYMNOSPERMS

+++ , Major constituent; ++ , present in some quantity; + , present as trace only; — , not present; a blank space indicates that the respective estimation was not done

Species	Nitrate in Leaf Extract	Total Nitrogen in Sap ($\mu\text{g/ml}$)	Sap Constituents														
			Nitrate	Aspartic Acid	Asparagine	Glutamic Acid	Glutamine	Alanine	Methionine and/or Valine	γ -Aminobutyric Acid	Tyrosine	Leucine	Serine	Threonine	Citrulline	Allantoin	Allantoic Acid
<i>Ginkgo biloba</i> L. <i>Taxus cuspidata</i> Sieb. & Zucc. <i>Podocarpus totara</i> D. Don <i>Cephalotaxus harringtonia</i> Koch <i>Agathis australis</i> Salisb. <i>Pinus radiata</i> D. Don <i>Sequoia gigantea</i> Deene. <i>Chamaecyparis lawsoniana</i> Parl. <i>Ephedra</i> sp.	+	68	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+
	+	67	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+
	+	44	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	—	67	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	—	47	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	—	16	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+
	—	17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	—	61	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+																
	+																

species of dicotyledonous plants from which xylem sap was tested for nitrate none could be detected.

Eighty-seven of the dicotyledonous species had glutamine as a major constituent of the xylem sap while 34 had asparagine. (These figures include some saps which had both amides present in major amounts.) Citrulline was present in sap from

TABLE 4

PRESENCE OF CITRULLINE, ALLANTOIN, AND ALLANTOIC ACID IN XYLEM SAP OF PLANTS

+++ , Major constituent; ++ , present in some quantity; + , present as trace only; — , not present

Species	Citrulline	Allantoin	Allantoic Acid
<i>Juglans regia</i> L.	++	—	—
<i>Alnus glutinosa</i> (L.) Gaertn.	+++	—	—
<i>Nothofagus fusca</i> Oerst.	+++	—	—
<i>Ulmus glabra</i> Huds.	—	—	+
<i>Atriplex</i> sp.	—	+	—
<i>Bougainvillea glabra</i> Chois.	—	+	+
<i>Phytolacca octandra</i> L.	+	+	—
<i>Annona cherimola</i> Mill.	+++	—	—
<i>Persea americana</i> Mill.	++	—	+++
<i>Romneya coulteri</i> Harvey	—	+	++
<i>Raphanus raphanistrum</i> L.	—	—	+
<i>Escallonia macrantha</i> Hook. & Arn.	—	—	++
<i>Liquidambar styraciflua</i> L.	+	—	+
<i>Platanus orientalis</i> L.	—	+	—
<i>Albizia lophantha</i> Benth.	+	+	+
<i>Homolanthus polyandrus</i> Cheesem.	—	+	—
<i>Acer pseudo-platanus</i> L.	—	+	+++
<i>Aesculus indica</i> Colebr.	—	+	+++
<i>Alectryon excelsum</i> Gaertn.	—	+	+++
<i>Hoheria populnea</i> A. Cunn.	—	+	—
<i>Carica papaya</i> L.	—	—	++
<i>Diospyros kaki</i> L.f.	+++	—	—
<i>Calystegia sepium</i> R. Br.	—	—	+
<i>Cobaea scandens</i> Cav.	—	—	++
<i>Ehretia acuminata</i> R. Br.	—	+	—
<i>Avicennia officinalis</i> L.	—	+	—
<i>Leonotus leonurus</i> R. Br.	—	—	+
<i>Cyphomandra betacea</i> Sendt.	—	+	+
<i>Arundinaria</i> sp.	—	+	+
<i>Chamaecyparis lawsoniana</i> Parl.	+++	—	—

29 species (out of 103 specifically tested) and in five of these it was present as a major constituent. Allantoin or allantoic acid or both were present in 23 species. These results are detailed in Table 4. Traces of urea were present in some of the saps containing these ureides.

A few hydrolysed sap samples were examined chromatographically. Each showed the appearance of glycine, cystine, and cysteic acid, none of which was

previously present, thus indicating the possible presence of a peptide in the sap. In this connection it should be pointed out, however, that Osono, Mukai, and Tominaga (1955) have detected breakdown of methionine to glycine and other acids on hydrolysis in presence of sugars.

IV. DISCUSSION

The plants examined in this work were all selected as morphologically capable of yielding xylem sap under the vacuum-extraction technique. Efforts were made to include representatives of as many families as possible.

Nitrate was present in a small number of leaf and sap samples. There was not complete correspondence between these—some plants with nitrate in their leaves had no detectable nitrate in the xylem sap. However, this sap was a dilute liquid and a low concentration of nitrate could well lead to an accumulation in the foliage. It would seem that an appreciable number of the plants sampled were similar to the Rosaceae, in which nitrate does not normally occur in the foliage. Low concentrations of nitrate were found in leaves of other species but in only a few were higher concentrations found.

In all xylem sap samples a mixture of organic nitrogenous substances was found. Even in those few species with appreciable nitrate in the sap considerable amounts of organic nitrogenous compounds also occurred. No quantitative measurements have been made on individual sap constituents but it was apparent from qualitative chromatography that, in all cases, the range of substances identified would account for most of the nitrogen in the sap.

A striking feature of the results was the quantitative importance of aspartic and glutamic acids and particularly of their amides. Glutamine was more abundant than asparagine in most species but it must be remembered that only one sampling was made on each species and there may well be changing ratios during the season. In only a few samples were neither glutamine nor asparagine found to be major constituents.

Other amino acids (alanine, methionine or valine or both, γ -aminobutyric acid, tyrosine, leucine, serine, threonine) were variously present in low concentration in all saps examined. This collection of compounds bears a striking resemblance to those already known to be present in xylem sap from the Rosaceae.

In a number of the species citrulline was present as a major sap constituent. Citrulline has not been recorded widely in plants but Miettinen and Virtanen (1952) found it as a major constituent of root nodules of *Alnus*. More recently Reuter and Wolfgang (1954) found citrulline in bleeding sap from a number of members of the Betulaceae. The present work has shown that, while this compound was not widely distributed among the species studied, it was of considerable quantitative importance in the xylem sap of several species.

The occurrence of allantoin or allantoic acid or both is of interest. While the presence of these compounds in some plants has long been known—a review of earlier work is given by Tracey (1955)—they have received comparatively little attention

until recently. Mothes and Engelbrecht (1952) have claimed that in *Acer* spp. these compounds are of major importance as translocatory forms of nitrogen. Reuter and Wolfgang (1954) have shown that allantoic acid is present in bleeding sap from *Aesculus hippocastanum*. The present work has shown that one or other of these compounds was present in sap from species of 23 dicotyledonous families and one monocotyledonous species. In some of these they were present as major constituents but in others as trace constituents only.

In animal metabolism allantoin and allantoic acid are excretory compounds being regarded as end-products of purine metabolism. In plants it is difficult to visualize these compounds as breakdown products of purines. Mothes (1953) regards them as synthetic products which in some plants are of primary importance in the storage and translocation of nitrogen.

The present work has confirmed that allantoin and allantoic acid are important translocatory compounds in a number of groups of plants. They are always accompanied in xylem sap, however, by a number of other amino acids or amides and these latter are usually more important quantitatively. In only three species (*Persea*, *Acer*, and *Alectryon*) did allantoic acid appear to be more important than the amides. Further elucidation of the relative importance of these compounds must involve examination of samples taken on more than one occasion through the growing season.

In recent years increasing attention has been paid to the differing roles of root and leaf in nitrogen metabolism. Mothes (1953) pointed out the differing capabilities of root and shoot in the synthesis of different materials. Working with isotopic nitrogen Turchin, Guminskaya, and Plyshevskaya (1955) showed that in young oat plants mineral nitrogen entering the plant was used rapidly in the roots for synthesis of amino acids. In the apple Bollard (1953*b*, 1957*a*) showed that nitrogen passing from root to shoot was largely in organic forms and that assimilation of inorganic nitrogen appeared to take place in the root system. In the apple there is some evidence that leaves cannot metabolize nitrate but nothing is known of the specific requirements of the shoot in terms of organic nitrogenous compounds. It may be that all the shoot requires is a supply of organic nitrogen, or it may be that all or some of the specific compounds supplied by the root are essential for shoot development.

Though the course of assimilation of inorganic nitrogen in roots of the plants studied is varied, many of the plants produced glutamine or asparagine or both as major sap constituents, together with a range of amino acids in lesser amounts. The presence of a similar type of peptide-like substance in, at least, some saps is a further point of similarity in the mode of assimilation. However, no description of this process can be considered complete until account is taken of the formation of citrulline, allantoin, and allantoic acid.

It is of interest that Tolbert and Wiebe (1955) have reported the presence of organic phosphorus compounds in xylem sap indicating that partial assimilation of this element may also occur in the root system.

V. ACKNOWLEDGMENTS

The author is grateful for the assistance of Miss J. M. Dingley, Mrs. R. F. Elliot, Dr. J. A. Rattenbury, and Dr. H. M. Mouat for assistance with location, identification, and collection of the plants used.

The assistance of N. A. Turner of this Research Station in the laboratory work is also acknowledged.

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PHYSIOLOGY OF PEA FRUITS

III. CHANGES IN STARCH AND STARCH PHOSPHORYLASE IN THE DEVELOPING SEED

By DONFLA H. TURNER* and J. F. TURNER*

[Manuscript received March 18, 1957]

Summary

During the growth of pea seeds there is a period in which the starch content rises rapidly and the sucrose content falls. This paper describes a study of the control of this starch synthesis. A method was developed for the assay of starch phosphorylase in peas and the changes in enzyme activity during development of the pea seed were followed. Over most of the period of development a linear relationship existed between the rate of starch synthesis and starch phosphorylase activity. It is suggested that the activity of starch phosphorylase may be a major factor in controlling the rate of starch formation in the pea. The rate of starch synthesis began to decrease when the pea ceased to gain water and the linear relationship then no longer held. The bearing of these observations on sugar-starch relationships and on the general problems of growth is discussed.

I. INTRODUCTION

It is well recognized that starch and sugars (especially sucrose) are closely related in many plant tissues. Advances in knowledge of the enzyme systems concerned in starch (Hanes 1940) and sucrose metabolism (Turner 1953, 1954; Cardini, Leloir, and Chiriboga 1955; Leloir and Cardini 1953, 1955) have indicated the probable mechanisms of carbohydrate changes in plant tissues. There is, however, little information on the control of the direction and extent of the starch and sugar syntheses and of starch-sugar transformations.

It was considered that the developing pea might provide a suitable material for the study of changes in carbohydrate metabolism during growth and of the factors controlling starch synthesis. During development of the pea seed there is a rapid rise in the starch content (Bisson and Jones 1932; McKee, Robertson, and Lee 1955) and this is accompanied by a fall in sugars. The change from a sweet, succulent pea to a seed of high starch content is of considerable commercial importance. The content of alcohol-insoluble solids (of which starch is a major component) has been used as a measure of maturity in peas (Kertesz 1935) and correlates well with physical determinations of hardness (Lynch and Mitchell 1950).

The present experiments are concerned with the changes in starch content and starch phosphorylase activity during the growth of pea seeds. Changes in certain other constituents were followed simultaneously.

II. MATERIALS AND METHODS

(a) Sampling of Peas

Peas (*Pisum sativum* L., var. Canner's Perfection) were taken from crops at Hawkesbury Agricultural College, Richmond, N.S.W. In order to provide pods

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of the same age, fully opened flowers were tagged on one day in each season. The dates of tagging were October 14, 1954, and September 25, 1955. Subsequently on specified days at 7 a.m. a minimum of 150 tagged pods was picked at random. Two hours later the peas were cut from the hulls, mixed, and samples taken for analysis.

(b) *Analytical Methods*

Fresh weight, dry weight, and moisture content were determined by the methods described by Turner (1949). Starch was determined by the methods of Nielsen and Gleason (1945) and Pucher, Leavenworth, and Vickery (1948). These methods gave similar results but difficulty was encountered in hydrolysing the starch by the method of Pucher *et al.* The values reported here were obtained by the method of Nielsen and Gleason. Sucrose was determined by a method to be described (Turner, Turner, and Lee, unpublished data).

(c) *Assay of Starch Phosphorylase*

The assay was based on the method of Green and Stumpf (1942), with the addition of molybdate to inhibit phosphatase as suggested by Bailey, Thomas, and Whelan (1951). Phosphoglucomutase did not interfere during the assay period. The plant extract was incubated with glucose 1-phosphate, a starch primer, and ammonium molybdate. The inorganic orthophosphate liberated after a standard time was estimated and taken as a measure of starch phosphorylase activity.

(i) *Reagents*.—Glucose 1-phosphate was prepared by an unpublished method of Professor C. S. Hanes and Dr. R. Hill. A commercial soluble starch was used as primer for the reaction. The soluble starch could be replaced by potato or pea starch.

(ii) *Preparation of Enzyme Extracts*.—The standard assay enzyme was prepared as follows:

Peas (10 g) were finely ground with 12 ml water and acid-washed sand. The ground material was centrifuged at 1000 *g*, the supernatant filtered through cotton wool, and centrifuged at 20,000 *g* for 20 min at 0°C. To 10 ml supernatant was added 40 ml cold saturated ammonium sulphate, pH 6.0. After standing for 1 hr at 0°C the precipitate was collected by centrifuging at 20,000 *g* for 7 min at 0°C, taken up in water, and diluted to the required volume.

Partially purified starch phosphorylase was obtained by repeated fractional ammonium sulphate precipitation of pea extracts.

(iii) *Measurement of Enzyme Activity*.—A mixture of 0.25 ml 0.6M citrate buffer (citric acid–NaOH), pH 6.0, 0.1 ml 0.018M ammonium molybdate, and 1.0 ml enzyme extract was incubated at 30°C. After 15 min, 0.25 ml 5 per cent. (w/v) soluble starch and 0.2 ml 0.26M glucose 1-phosphate, pH 6.0, were added and samples taken for analysis at 0 and 20 min. The rate of reaction was constant during the 0–20-min period. Assays for starch phosphorylase on replicate samples of peas agreed within 5 per cent.

Inorganic phosphate was determined on trichloroacetic acid extracts of the enzyme digests by the method of Allen (1940). Maltose and glucose were detected

by running trichloroacetic acid extracts on paper chromatograms using *n*-propanol-ammonia-water (80 : 15 : 5 v/v) as solvent and the silver spray of Trevelyan, Procter, and Harrison (1950).

One unit of enzyme is defined as the amount which catalyses the liberation of 0.05 mg inorganic phosphorus per 1.8 ml digest in 20 min at 30°C. This represents 3.1 per cent. of the glucose 1-phosphate supplied and digests containing 1-4 units of enzyme were used. Within this range the activity of the partially purified enzyme preparations and of the standard assay preparations was directly proportional to the concentration of enzyme.

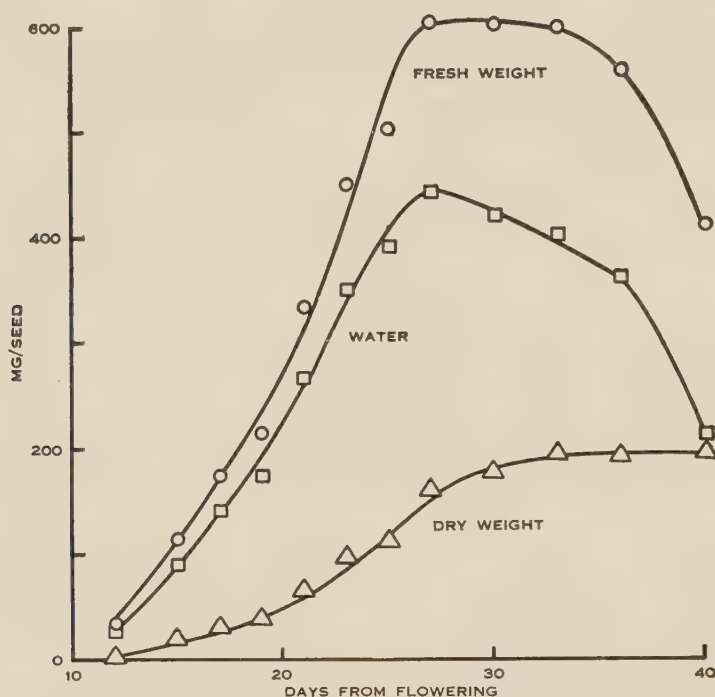


Fig. 1.—Changes in fresh weight, dry weight, and water in pea seeds with time from flowering.

(iv) *The Presence of Phosphatase and Amylases.*—When pea enzyme extracts were allowed to act on glucose 1-phosphate there was some phosphatase action which was detected by the production of glucose. The phosphatase was completely inhibited by $1 \times 10^{-3}M$ ammonium molybdate. This concentration of molybdate had no effect on the starch phosphorylase activity of either the partially purified or standard assay enzyme preparations. A figure for phosphatase activity was obtained from the difference in phosphate production between digests with and without molybdate. These values must be considered as approximate as Porter (1953) has observed that phosphatase activity may be depressed by high phosphorylase levels.

A change in iodine colour which was observed in digests containing starch and the standard enzyme assay extract suggested that α -amylase was present. Some

maltose was produced in similar digests indicating β -amylase activity. β -Amylase is absent from most starch-bearing leguminous seeds (Peat 1951). The use of mercuric chloride as an inhibitor of amylase activity was unsatisfactory as a routine procedure as the protein content of the assay extracts varied with the age of the peas. The concentration of mercuric chloride ($1 \times 10^{-4}M$) required to inhibit the amylases also caused some inhibition of starch phosphorylase. Porter (1950) has shown that β -amylase is an inhibitor of starch phosphorylase. The values quoted for starch phosphorylase may have been affected by the presence of β -amylase and should be

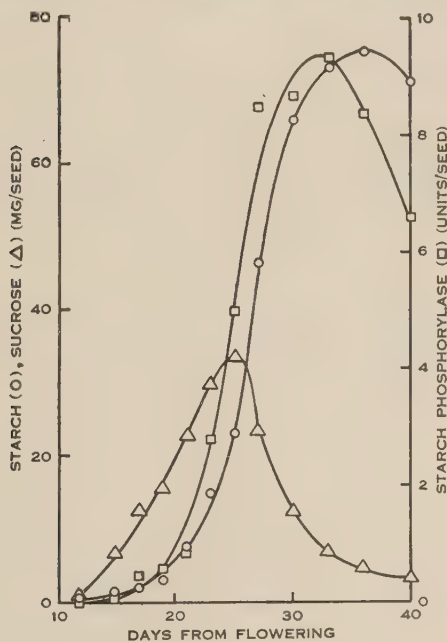


Fig. 2

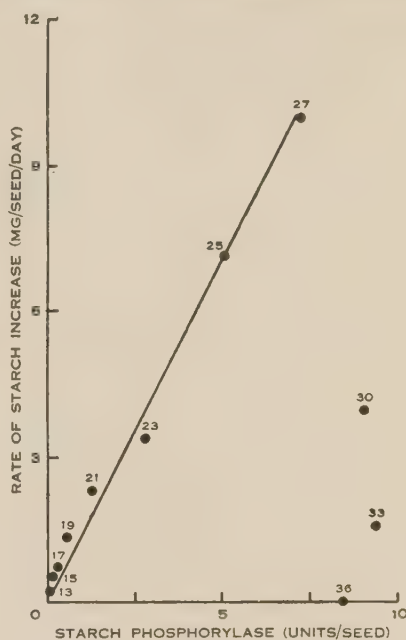


Fig. 3

Fig. 2.—Changes in starch, starch phosphorylase, and sucrose in pea seeds with time from flowering.

Fig. 3.—Relation between the rate of starch increase and starch phosphorylase activity at various times. The days from flowering are shown by the number adjacent to each point. The line was fitted visually.

considered as “apparent starch phosphorylase”. β -Amylase was very approximately estimated by following the rate of maltose formation in digests containing starch and the standard assay enzyme. Extracts of the enzyme digests were examined by paper chromatography and the maltose estimated visually.

III. RESULTS

Experiments were carried out in two successive seasons (1954 and 1955). The results were essentially the same in both seasons even though the maximum fresh weight per seed in 1955 was 50 per cent. above that of 1954. The activity of starch phosphorylase and also the starch content per unit fresh weight were similar in each

season. More samples were taken in 1955 and the results of this season are reported here.

From the time of the first sampling (12 days from flowering) fresh weight per seed increased steadily until 27 days (Fig. 1) and remained constant until 33 days. Fresh weight then decreased. The dry weight per seed increased until 30 days from flowering and thereafter changed very little; the dry weight did not fall at any stage in the experiment. Water per seed increased until 27 days from flowering and then decreased until 40 days when the water content was less than half the value attained at 27 days. McKee *et al.* (1955) also observed that the pea seed failed to accumulate water after a certain stage in development.

The changes in starch content per seed (Fig. 2) are in general agreement with results previously reported by Bisson and Jones (1932) and McKee *et al.* (1955). The starch content was very low at 12 days from flowering and increased slowly over the next 9 days. About 21 days after flowering a phase of rapid starch synthesis commenced and continued until 30 days. Subsequent changes in starch content were small. The changes in starch phosphorylase activity (Fig. 2) were approximately parallel to those in starch content.

By fitting tangents to the smoothed curve shown in Figure 2, the daily rates of starch synthesis were calculated. In Figure 3, these rates are plotted against the starch phosphorylase activities on the corresponding days, read from the smoothed curve in Figure 2. A linear relationship was obtained until 27 days from flowering after which time the remaining points were not linear. This departure from linearity coincides with the cessation of water increase.

The sucrose content per seed rose from the first sampling until 25 days after flowering and subsequently decreased rapidly (Fig. 2). Changes in sugars will be considered in detail in a subsequent publication.

Phosphatase activity rose until 23 days from flowering and then fell to a low level. β -Amylase activity appeared to be relatively high in the initial period to 23 days but subsequently decreased markedly.

IV. DISCUSSION

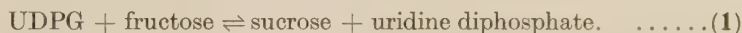
It is generally considered that starch phosphorylase is responsible for starch synthesis in plants. In some plant tissues, however, starch is formed when the ratio of inorganic phosphate to glucose 1-phosphate appears to be unfavourable for the production of starch. On the basis of this evidence Ewart, Siminovitch, and Briggs (1954) concluded that the phosphorylase reaction was not mediating starch synthesis. This conclusion is doubtful as the results of gross analyses of tissues may bear little relation to the actual concentration of reactants at the site of synthesis within the cell. This aspect has been discussed by Trevelyan, Mann, and Harrison (1954).

Sucrose is probably the main carbohydrate transported into the pea and therefore the main raw material for starch formation. It has been found in this Laboratory (Turner *et al.*, unpublished data) that the concentration of hexoses is not more than 5 per cent. of the sucrose concentration during the main part of the growing period of the pea. From the data of McKee *et al.* (1955) it can be calculated that the total phosphorus in the pea seed at 40 days after flowering is only 3.8 per cent. of

that which would be produced (as inorganic orthophosphate) by the observed formation of starch by the phosphorylase reaction. This indicates either that practically all the phosphorus entering the seed is re-exported or, more likely, that the amounts of hexose phosphates transported into the pea are too low to make a significant contribution to starch formation.

The rapid rise in starch content and corresponding fall in sucrose are the main points taken for consideration in the present discussion. The sucrose content increased from the first sample until 25 days from flowering. Starch was then increasing and, although sucrose was probably still being imported at approximately the same rate, starch synthesis was so intense that the level of sucrose fell. These trends continued until 30 days from flowering after which time there was little increase in dry weight of the seed.

There is evidence (Turner 1953, 1954; Cardini *et al.* 1955) that sucrose may be synthesized in pea extracts from uridine diphosphoglucose (UDPG) and fructose by reaction (1):



UDPG may be formed by reaction (2) which has been observed in extracts from yeast and animal tissues (Munch-Petersen *et al.* 1953; Mills, Ondarza, and Smith 1954):



The degradation of sucrose by a reversal of these reactions would yield glucose 1-phosphate and fructose. An active fructokinase which catalyses the phosphorylation of fructose to fructose 6-phosphate has been observed in pea extracts in this Laboratory and by Medina and Sols (1956). Both phosphohexoisomerase and phosphoglucomutase occur in peas (Hanes 1940) and these enzymes make possible the conversion of fructose 6-phosphate to glucose 1-phosphate. Thus sucrose may be completely converted to glucose 1-phosphate, the substrate for starch phosphorylase.

In the initial period of growth of the pea (up to 21 days from flowering) the level of starch phosphorylase was low and there was little starch synthesis. Both phosphatase and β -amylase activities were relatively high up to 23 days from flowering. Phosphatase may have depressed starch synthesis by removing part of the available glucose 1-phosphate and β -amylase may have inhibited starch phosphorylase (Porter 1950). There was no evidence that the amylases degraded starch in the developing pea seed. Ono (1955) has observed that a decrease in β -amylase activity in the radish coincides with the period of starch formation.

After 21 days from flowering the starch phosphorylase activity increased rapidly and an acceleration of starch synthesis ensued. Over most of the period of development of the pea there was a linear relationship between the rate of starch formation and the starch phosphorylase activity. This suggests that the level of starch phosphorylase may be a major factor controlling starch synthesis in the peas under investigation.

The lack of any direct relationship between starch and starch phosphorylase after 27 days from flowering is interesting. The departure from linearity coincides with the cessation of net water uptake by the seed and may be closely related to this phenomenon. After 27 days the water content of the seed decreased and the transformation to the dry seed began. It is apparent that a point must be reached when starch synthesis ceases, perhaps ultimately due to substrate limitation as well as to the desiccation of the regions of synthesis; at this stage considerable amounts of starch phosphorylase are still present. Starch phosphorylase may be readily demonstrated in dried pea seeds (Hanes 1940).

The concentration of glucose 1-phosphate necessary to maintain the observed rate of starch synthesis in the pea may be approximately estimated. Rowan (unpublished data) found that the peak concentration of hexose monophosphates during the period of active starch synthesis in the peas under investigation was approximately $2 \mu\text{moles/g}$ fresh weight. Glucose 1-phosphate is unlikely to comprise more than 5–10 per cent. of the hexose monophosphate fraction. From the enzyme data obtained in this investigation and assuming a mean growing temperature for the peas of 15°C (with a Q_{10} of 2 for starch phosphorylase) it can be calculated that the glucose 1-phosphate concentration needed for the observed rate of starch formation must be of the order of $5 \mu\text{moles/g}$ fresh weight. This value is minimal as it is derived from initial velocities in the enzyme assays and assumes an inorganic phosphate concentration approximating to zero. It appears, therefore, that the concentration of glucose 1-phosphate at the site of starch synthesis is considerably higher than the content found by analysis of the whole tissue, and that there are regions of high concentration within the cells.

There is little information on the factors which may control or influence the absolute level of starch phosphorylase. The possibility of adaptive enzyme formation in response to the presence of glucose 1-phosphate cannot be excluded. The change may also be the response to some hormone variation or genetic characteristic which is part of the normal growth pattern. The results obtained may have some interesting implications regarding the general problems of growth in plants and the extent to which the activity of enzyme systems may control syntheses and metabolism as a whole.

V. ACKNOWLEDGMENTS

The work described in this paper was carried out as part of the joint research programme of the Division of Food Preservation and Transport, C.S.I.R.O., and of the Botany School, University of Sydney. The authors wish to express their indebtedness to Mr. J. B. Lee and Mr. J. Smydzuk for carrying out some of the analyses; to Mr. L. J. Lynch, Mr. R. S. Mitchell, and Mr. G. G. Coote for their helpful advice; to Hawkesbury Agricultural College for the supply of peas; to Dr. R. N. Robertson for his interest during the course of the investigation; and to Dr. J. R. Vickery, Chief, Division of Food Preservation and Transport, and Professor R. L. Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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RESPIRATION OF THE SPADIX FROM THE AROID *ZANTEDESCHIA AETHIOPICA* SPRENG.

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[Manuscript received December 10, 1956]

Summary

Spadix tissue of a number of aroid species has been shown to be an outstanding exception to the general pattern of plant respiration. In the work reported here the respiration of whole tissue and mitochondria from the spadix of another aroid species *Zantedeschia aethiopica* Spreng. was studied. The respiration of both whole tissue and mitochondria was found to be strongly inhibited by the metal-complexing agents cyanide and azide. Cytochrome oxidase and cytochromes *b* and *c* were spectroscopically identified in mitochondrial suspensions and cytochrome oxidase activity was demonstrated in extracts. The results indicate that the respiratory characteristics of the *Zantedeschia* spadix tissue are similar to those of most other plant tissues. Such characteristics are, however, in marked contrast to the respiratory pattern reported for other aroid spadices.

I. INTRODUCTION

It is apparent that aroid spadix tissue provides an outstanding exception to the general pattern of plant respiration. This tissue is characterized by an exceptionally rapid respiration rate (500–3000 $\mu\text{l O}_2/\text{g fresh wt./hr}$). In 10 species reported (van Herk 1937; James and Beevers 1950; van Norman 1955; Yocum and Hackett 1955) this respiration was unaffected or only slightly inhibited by cyanide even at a concentration as high as 10^{-2}M . In contrast to cytochrome oxidase-mediated respiration, the respiration rate of these tissues is apparently directly dependent on oxygen tensions up to 100 per cent. Such exceptions to the usual respiratory pattern of plants are of considerable importance at a time when there is a growing realization of the marked overall uniformity of fundamental biochemical pathways.

This paper is concerned with the respiratory properties of the whole tissue and mitochondria of the spadix of the aroid *Zantedeschia aethiopica* Spreng. (arum lily). The respiratory characteristics of this tissue are discussed in relation to those of other aroid species, and to the general pattern of plant respiration.

II. MATERIALS

Spadix tissue of *Z. aethiopica* was used. Material was chosen at a stage of maturity equivalent to the δ - ϵ stage of the James and Beevers (1950) development classification. The spadix is differentiated into a central rachis, about which are compactly arranged the yellow male flower structures. The respiratory properties of both the whole spadix and these separate portions were studied.

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III. METHODS

(a) *Preparation of Tissue Slices*

Slices (approx. 1 mm thick) were cut from the whole spadix and washed with three changes of distilled water. The randomized slices were blotted dry on filter paper, weighed into equal lots ($1\text{ g} \equiv 125\text{ mg}$ dry weight), and added to manometer flasks. To determine the error of such sampling the respiration rates of five replicate experiments were examined. The slices were suspended in 0.066M phosphate buffer (pH 5.0). The standard deviation calculated was 1.5 per cent. of the arithmetic mean. In experiments in which the differentiated portions of the spadix were studied, male flower segments ($0.5\text{ g} \equiv 0.076\text{ mg}$ dry weight), which were easily removed from the rachis, were used as such after an initial washing with distilled water. Slices of the rachis ($0.5\text{ g} \equiv 0.058\text{ mg}$ dry weight) were prepared in a similar manner to the whole spadix slices.

(b) *Preparation of Cytoplasmic Particles*

Whole spadix tissue (15 g) or its differentiated parts were washed in distilled water, sliced, then ground in a mortar with sand and 50 ml of 0.5M sucrose. The homogenate was strained through muslin, and the filtrate centrifuged for 3 min at 2000 *g*. The supernatant was then centrifuged at 16,000 *g* for 10 min. The residue obtained was washed twice by resuspension in 0.5M sucrose and recentrifugation at 16,000 *g*. The final residue was suspended in 0.5M sucrose. Aliquots (0.4–0.6 mg total nitrogen) of this suspension were used in manometric experiments. All operations were carried out between 0 and 2°C. The addition of "Versene" to the preparation media had no detectable effect on the activity of the isolated particles.

(c) *Application of Inhibitors*

Mixtures of calcium hydroxide and calcium cyanide (Robbie and Leinfelder 1945; Robbie 1948) were used to absorb carbon dioxide in the experiments on the effects of cyanide on respiration. When azide was used as an inhibitor, the tissue was allowed to incubate with the inhibitor for 30 min before potassium hydroxide was added to the centre well. This was done to minimize the effects of the diffusion of azide into the alkali of the centre well.

Oxygen uptake was measured at 30°C by the standard Warburg manometric technique. Spectroscopic examination of mitochondrial suspensions was carried out with a Zeiss-Winkel microspectroscope equipped with a wavelength scale. Sodium hydrosulphite was used as a reducing agent. Total nitrogen was determined by Nesslerization of acid digests of mitochondrial suspensions. The coenzyme concentrate was prepared from dried brewer's yeast (Le Page and Mueller 1949). Cytochrome *c* was prepared by the method of Keilin and Hartree (1937) and standardized spectrophotometrically (Umbreit, Burris, and Stauffer 1951). Cytochrome oxidase activity was assayed according to Umbreit *et al.* (1951).

IV. EXPERIMENTAL

(a) *Respiration of Sliced Tissue*

The respiration of spadix tissue slices from all aroids so far examined has been remarkably resistant to cyanide, and, where tested, to azide (van Herk 1937; James

and Beevers 1950; James 1953; van Norman 1955). It is of interest therefore that slices from the spadix of *Z. aethiopica* show a quite different response to these inhibitors. Cyanide (10^{-3}M) caused 55 per cent. inhibition during the first hour and 89 per cent. during the third hour (Fig. 1). Azide (10^{-3}M) was an even more rapidly effective inhibitor resulting in an 83 per cent. inhibition during the first hour and an 87 per cent. inhibition during the third hour (Fig. 1).

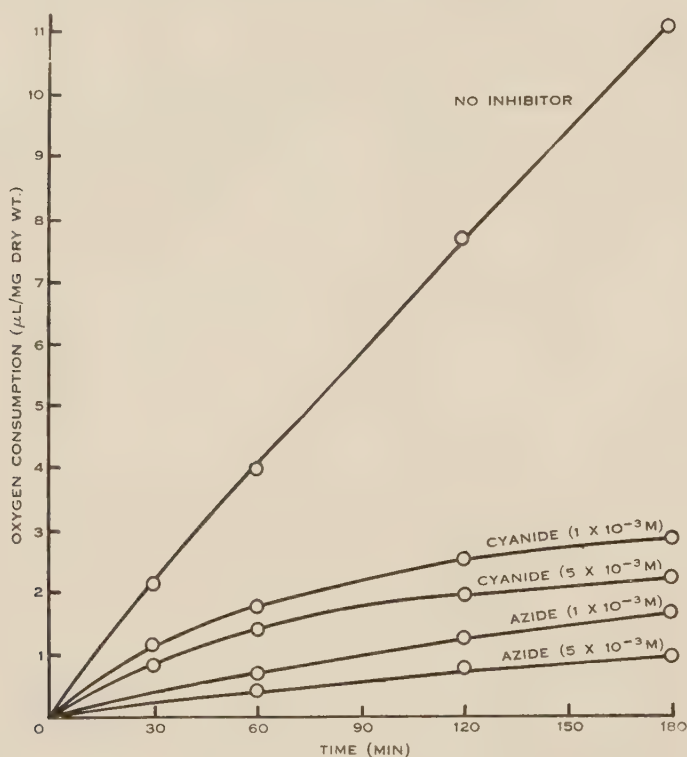


Fig. 1.—Effect of cyanide and azide on the respiration of *Zantedeschia* spadix slices. The control system contained tissue (1.0 g wet wt.) suspended in 0.06M phosphate buffer at pH 5.0.

The pH at which these ionic inhibitors are applied to plant tissue is an important factor in determining the rate at which they penetrate the tissue, and therefore the degree of inhibition observed (James 1953). In a separate experiment it was shown that the respiration of *Zantedeschia* spadix was only slightly inhibited after 2 hr by cyanide and azide ($5 \times 10^{-3}\text{M}$) at pH 7.0. At pH 5.0, however, azide exerts its inhibitory effect rapidly, but the effect of cyanide, although considerably more rapid than at pH 7.0, is still apparently limited during the first hour by its rate of penetration to the site of inhibition (Fig. 1).

A respiratory rate of $500 \mu\text{l O}_2/\text{g fresh wt.}/\text{hr}$ was recorded for *Zantedeschia* spadix tissue. Although this is not as high as the rates observed in some other spadix tissue (Yocum and Hackett 1955), it is, nevertheless, high compared with most plant tissues.

The spadix tissue, as is shown in Figure 1, had a basal respiration stable to cyanide. The respiration of both the male flower segments and the rachis contributed to this cyanide-stable portion of the respiration of the whole tissue. However, as is shown in Figure 2, the male flower portions had a somewhat larger cyanide-stable basal respiration (65 per cent. inhibition with $5 \times 10^{-3}M$ cyanide after 2 hr) than did the rachis (79 per cent. inhibition with $5 \times 10^{-3}M$ cyanide after 2 hr). The effect of azide on the respiratory rates of both the rachis and male flower portions

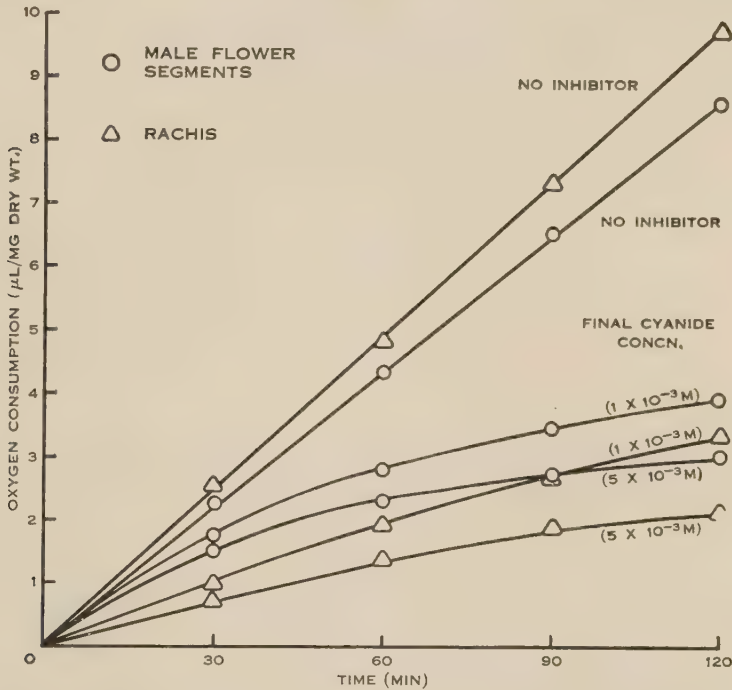


Fig. 2.—Effect of cyanide on the respiration of the differentiated portions of the *Zantedeschia* spadix. The control system contained tissue (0.5 g wet wt.) suspended in 0.06M phosphate buffer at pH 5.0. The final cyanide concentrations are as indicated.

was similar to that of cyanide. Azide ($10^{-3}M$), however, was a somewhat more efficient inhibitor than cyanide, causing, after 2 hr, 91 per cent. inhibition of rachis respiration, and 80 per cent. inhibition of the respiration of the male flower segments.

(b) Oxidative Activity of Isolated Mitochondria

Since it is well established that the enzymes responsible for the aerobic phases of respiration are localized in the mitochondria, it was of interest to examine mitochondria isolated from *Zantedeschia* spadix. Such cytoplasmic particles rapidly oxidized all the acids of the Krebs cycle (Table 1). Oxidation of pyruvate was dependent upon the concomitant oxidation of a small amount of one of the other acids of the cycle (Table 2). These findings are in agreement with Hackett and Simon (1954) for mitochondria isolated from *Arum* spadix.

The results shown in Tables 1 and 2 represent the activities of mitochondria isolated from the whole spadix tissue. Table 3 gives details of the oxidative activity

TABLE 1
OXIDATION OF KREBS-CYCLE ACIDS BY MITOCHONDRIA
ISOLATED FROM ZANTEDESCHIA SPADIX

The complete system contained mitochondrial suspension; substrate (2×10^{-2} M); phosphate buffer pH 7.0 (2.5×10^{-3} M); sucrose (5×10^{-1} M); MgSO_4 (1×10^{-3} M); adenosine monophosphate (1×10^{-3} M); yeast nucleotide (1 mg). Total volume 2.0 ml

Experiment	Substrate	Oxygen Uptake ($\mu\text{l O}_2/\text{mg N}/30 \text{ min}$)
1	None	27
	Succinate	226
	α -Ketoglutarate	102
2	None	22
	Citrate	170
	Malate	92

of mitochondria isolated from the two portions of the spadix, the rachis and the male flower segments. Mitochondria isolated from the rachis were the more active on a

TABLE 2
PYRUVATE OXIDATION BY ZANTEDESCHIA SPADIX MITOCHONDRIA CATALYSED BY KREBS-CYCLE
ACIDS

Complete system as in Table 1. Substrate concentration as indicated

Experiment	Substrate	Concn. (M)	Oxygen Uptake ($\mu\text{l O}_2/\text{mg N}/30 \text{ min}$)	
			Krebs-cycle Acid	Krebs-cycle Acid + 0.02M Pyruvate
1	None		23	—
	Pyruvate	2×10^{-2}	24	—
	Succinate	1×10^{-3}	73	125
	Malate	1×10^{-3}	48	77
2	None		21	—
	Pyruvate	2×10^{-2}	24	—
	α -Ketoglutarate	1×10^{-3}	37	86
	Citrate	1×10^{-3}	36	74

Q_{O_2} (N) basis but those from the male flower segments were responsible for most of the endogenous oxygen consumption. When mitochondria were prepared from

whole tissue the endogenous oxygen consumption was not reduced by repeated washing of the isolated particles and was completely insensitive to cyanide (Table 4). The results recorded in Table 3 indicate that the mitochondria from the male flower

TABLE 3
OXIDATIONS OF KREBS-CYCLE ACIDS BY MITOCHONDRIA ISOLATED FROM THE
DIFFERENTIATED PARTS OF ZANTEDESCHIA SPADIX
Complete system as in Table 1

Substrate	Oxygen Uptake ($\mu\text{l O}_2/\text{mg N}/30 \text{ min}$)	
	Male Flower Segments	Central Rachis Tissue
None	29	11
Succinate	145	409
α -Ketoglutarate	80	152
Citrate	93	258
Malate	80	134

segments were responsible for most of this cyanide-stable endogenous oxygen consumption. Such an observation is in keeping with the results expressed in

TABLE 4
INHIBITION OF MITOCHONDRIAL OXIDATIONS BY CYANIDE: WHOLE SPADIX
Complete system as in Table 1

Experiment	Substrate	Oxygen Uptake ($\mu\text{l O}_2/\text{mg N}/30 \text{ min}$)	
		Krebs-cycle Acid	Krebs-cycle Acid + 0.001M Cyanide
1	None	16	18
	Succinate	130	14
	α -Ketoglutarate	74	16
2	None	22	22
	Citrate	84	20
	Malate	67	24

Figure 2. Although the endogenous oxygen consumption of the isolated mitochondria was cyanide-stable, oxidation of intermediates of the tricarboxylic acid cycle was completely inhibited by $1 \times 10^{-3}\text{M}$ cyanide (Table 4).

(c) *Demonstration of the Complete Cytochrome System in Mitochondria Isolated from Zantedeschia Spadix*

The cyanide sensitivity of mitochondrial oxidations (Table 4) suggests that the cytochrome system may well be operating in these particles. This has been shown by assaying the cytochrome oxidase activity of these particles and by spectroscopic identification of the three cytochromes.

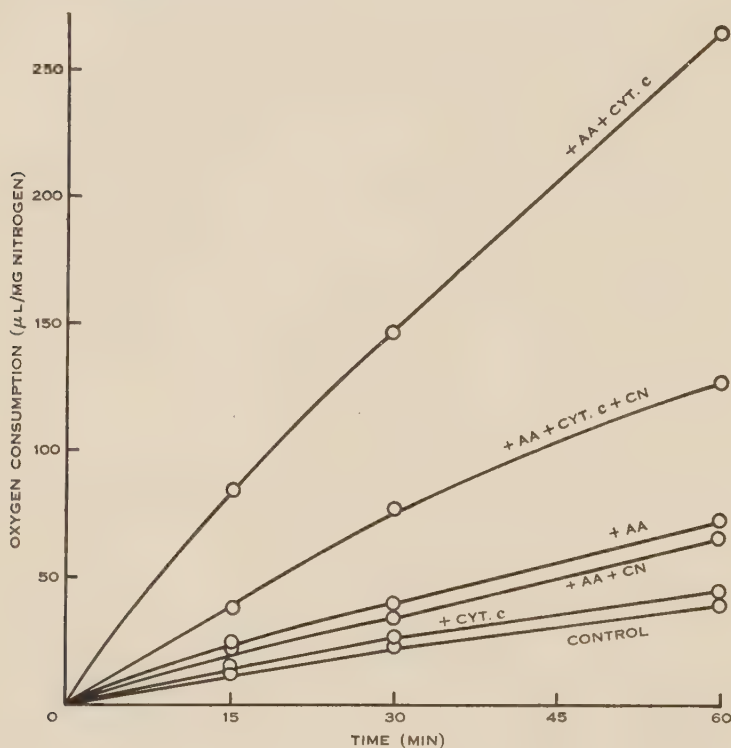


Fig. 3.—Cytochrome oxidase activity in a particulate preparation from *Zantedeschia spadix* mitochondria. The control system contained particulate enzyme (0.58 mg nitrogen), 0.025M phosphate buffer at pH 7.0, and 0.001M MgSO_4 . Additions were: Ascorbic acid (AA) ($1 \times 10^{-2}\text{M}$), cytochrome c (CYT. c) ($3 \times 10^{-5}\text{M}$), and hydrogen cyanide (CN) ($2.5 \times 10^{-4}\text{M}$). Total volume 2 ml.

(i) *Cytochrome Oxidase Activity of Zantedeschia Mitochondria*.—Mitochondria were prepared as described in Section III to the first washing stage, but the particles were then washed twice with distilled water. This procedure damages the structure of the mitochondria and, if cytochrome oxidase is operating, the particles will then show a requirement for cytochrome c. The oxidation of ascorbic acid by these particles was then examined, and, as is shown in Figure 3, this oxidation was markedly stimulated by the addition of cytochrome c. This cytochrome-mediated oxidation of ascorbic acid was cyanide-sensitive (60 per cent. inhibition at a cyanide concentration of $2.5 \times 10^{-4}\text{M}$).

(ii) *Spectroscopic Identification of the Cytochromes in Mitochondrial Suspensions.*—No absorption bands were observed in the untreated mitochondrial suspensions. When sodium hydrosulphite was added as a reducing agent three strong absorption bands appeared at 604, 563, and 553 m μ . These bands correspond to the α -absorption bands of the reduced forms of cytochrome oxidase and cytochrome *b* and *c* components respectively.

V. DISCUSSION

Plant tissues may vary considerably in respiratory rate. Variations from 10 to 500 μ l O₂/g fresh wt./hr are normally encountered. The total respiration of most plant tissues can be divided into two components defined by their behaviour to such metal-complexing agents as cyanide and azide. The component insensitive to such inhibitors is termed the "basal" respiration. This component characteristically contributes to only a small portion of the total respiration, and its magnitude is usually unaffected during changes in total respiration of a tissue. The major portion of the total respiration, which may be termed "non-basal", is inhibited by cyanide and may be extensively varied by different tissue treatments. The terminal oxidase system mediating this respiration has been identified in many tissues as the classical cytochrome system situated in the mitochondria.

The respiration of the spadix of a number of aroid species (van Herk 1937; James and Beevers 1950; van Norman 1955; Yocum and Hackett 1955) provides an outstanding exception to this pattern of plant respiration. In all species examined the respiration rate was shown to be exceptionally rapid in comparison with other plant tissues. This rapid respiration was apparently quite stable to such metal-complexing agents as cyanide and azide. In addition the respiration of tissue slices and homogenates from *Arum maculatum* L. was shown to be directly dependent upon oxygen tensions up to 100 per cent. (James and Beevers 1950). This is in sharp contrast to cytochrome-mediated respiration. Such observations have led to the general acceptance of a non-metallic oxidase as the functional terminal oxidase in these tissues. The behaviour of aroid spadix tissue to oxygen tensions has been further examined in *Peltandra* and a tropical *Philodendron* (Yocum and Hackett 1955). Sensitivity to oxygen tension was shown by these tissues only when the respiration was examined in a liquid phase. In air, the tissue slices showed a high affinity for oxygen and a low but measurable affinity for carbon monoxide. Such a combination of properties is, as the authors point out, not typical of any known non-metallic oxidase.

It is well established that the enzymes responsible for the aerobic phases of respiration are localized in the mitochondria, and it was thus of interest to examine the properties of mitochondria isolated from these aroids. Mitochondria which rapidly oxidized Krebs-cycle intermediates were readily isolated from the spadix of *A. maculatum* (Hackett and Simon 1954). Such oxidations were not inhibited by cyanide (James and Elliott 1955). Mitochondria were also isolated from the spadix of *Symplocarpus foetidus* Nutt. These particles rapidly oxidized Krebs-cycle acids and the absorption maxima of cytochrome *a*, *b*, and *c* components were spectroscopically identified (Bonner and Yocum 1956; Hackett 1956). The effects of cyanide and

azide on these mitochondrial oxidations were not typical of an oxidase system mediated by cytochrome oxidase alone. However, the cytochrome oxidase activity of these particles was itself sensitive to both inhibitors.

Bonner and Yocum (1956) indicated that the results were consistent with the view that the cytochrome *b* component which absorbs at 560 $m\mu$ is capable of linking dehydrogenases not only to cytochrome *c* but also directly to molecular oxygen. More recently, supporting evidence for this conclusion was obtained by Bendall and Hill (1956). They showed that tissue slices and mitochondrial suspensions prepared from *A. maculatum* contained a full complement of cytochromes in relatively high concentrations. In highest concentration was the cytochrome *b* component which absorbed at 560 $m\mu$ and was rapidly oxidized by molecular oxygen in the presence of cyanide. Although a normal cytochrome oxidase is present in these tissues it appears that the passage of electrons from substrate to molecular oxygen is, in part at least, diverted through this autoxidizable cytochrome *b* component in the intact tissue.

It is apparent then that spadix tissue from the aroids so far examined display respiratory behaviour quite atypical of most plant tissues.

The spadix of *Z. aethiopica*, however, presents a very different picture. Tissue slices from the spadix of this aroid, as from other aroids investigated, respired at a rapid rate (500 μ l O_2 /g fresh wt./hr), but *Zantedeschia* spadix respiration was quite sensitive to cyanide and azide (Fig. 1) provided that the inhibitor was applied at a sufficiently acid pH. Experiments using ionic inhibitors are significant only if the relationship between pH and penetration of the inhibitor has been considered. The respiration of the differentiated portions of the spadix was separately examined (Fig. 2). It was apparent that in the male flower segments the cyanide-stable basal respiration represented a greater portion of the total respiration than it did in the rachis.

Mitochondria isolated from *Zantedeschia* spadix rapidly oxidized tricarboxylic acid intermediates (Tables 1 and 2). Such oxidations were inhibited by cyanide (Table 4). Cytochrome oxidase activity was high in these particles (Fig. 3) and the typical α -absorption bands of cytochrome oxidase and cytochromes *b* and *c* were demonstrated. These mitochondria then show the general characteristics of mitochondria isolated from many plant sources but are different from those isolated from other aroid spadices.

Zantedeschia spadix therefore shows a normal respiratory pattern, and cytochrome oxidase is apparently the major terminal oxidase. The small component (10–15 per cent.) of the total respiration which is insensitive to cyanide and azide corresponds to the basal respiration characteristic of most plant tissues. Thus, although the respiration of *Zantedeschia* spadix is similar to that of the majority of plant tissues it is in marked contrast with that of spadices from other aroid species.

The possibility arises that there may be a correlation between the morphology and respiratory properties of these various spadix tissues. Of the types dealt with by James and Beevers (1950) (*Arum*, *Arisaema*, *Biarum*, *Amorphophallus*, and *Sauromatum*) each spadix has a barren terminal appendage (appendix) which was the portion

used as the experimental material. The basal portion of the spadix which bears bands of unisexual male and female flowers was not studied.

None of the other types which showed a respiration characteristic of the aroids described by James and Beevers (1950) has a similar spadix morphology. The spadix of *Symplocarpus foetidus* is completely covered with hermaphrodite flowers. *Peltandra* and *Philodendron* spadices bear unisexual flowers covering the whole surface, with the female flowers at the base and the male flowers on the upper portion.

The arrangement of flowers on the spadix of *Z. aethiopica* closely resembles that of *Peltandra* and *Philodendron*. The upper four-fifths of the total spadix length is covered by male flowers and the remainder by female flowers.

It is apparent that no obvious correlation exists between the morphology and the respiratory properties of the spadix tissues studied. The general morphology of the cyanide-insensitive types is not uniform nor are there significant differences between this group and *Z. aethiopica* which has a normal cyanide-sensitive respiration.

VI. ACKNOWLEDGMENTS

This work was carried out partly in the Department of Biochemistry, University of Sydney, and partly in the Plant Physiology Unit of the Botany School, University of Sydney, and of the Division of Food Preservation and Transport, C.S.I.R.O. It was supported by a University of Sydney Research Grant.

The authors wish to express their thanks to Dr. R. N. Robertson, Dr. F. E. Huelin, and Mr. K. T. Glasziou for helpful criticism of the manuscript.

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THE INFLUENCE OF PROGRESSIVE INCREASES IN TOTAL SOIL MOISTURE STRESS ON TRANSPIRATION, GROWTH, AND INTERNAL WATER RELATIONSHIPS OF PLANTS

By R. O. SLATYER*

[Manuscript received March 26, 1957]

Summary

The responses of tomato (*Lycopersicon esculentum* (Mill.), privet (*Ligustrum lucidum* Ait.), and cotton (*Gossypium barbadense* L.) to conditions of increasing total soil moisture stress were measured in terms of vegetative growth, stem elongation, transpiration, leaf turgor, diffusion pressure deficit, and osmotic pressure.

The response pattern of each species showed a close relationship to water stress, and in each species growth (as total dry weight) did not continue beyond a stress value such that there was zero turgor pressure in the tissue of adult leaves. In privet and cotton, stem elongation also ceased at this value; in tomato it continued until higher stress levels developed. As permanent wilting of the leaves was also associated with the point of zero turgor pressure, these results indicate that the permanent wilting percentage of any one soil is determined by the osmotic characteristics of the plant under study rather than by any soil characteristic. Permanent wilting occurred at soil water contents of 11.8, 9.7, and 10.2 per cent. in tomato, privet, and cotton respectively, equivalent to total soil moisture stress values of 20, 38, and 48 atm. The soil water content at 15 atm stress was 12.2 per cent.

At high stress levels there was strong evidence of nocturnal absorption of water directly from the atmosphere, and its accumulation in the tissues of the shoots of two of the species. Although a diffusion gradient appeared to exist favouring movement of water out of the plant into the soil no evidence of such movement was obtained. This is attributed to the development of discontinuity in root-soil contact.

I. INTRODUCTION

There is now general agreement that soil water becomes progressively less available to plants as total soil moisture stress (TSMS) increases (Richards and Wadleigh 1952). Evidence to the contrary (Veihmeyer and Hendrickson 1950; Veihmeyer 1956) is based almost entirely on field experiments where availability is complicated by uneven root distribution, and the measurement of plant response cannot be conducted accurately or readily interpreted. Depending on the sensitivity of stomatal closure, the effectiveness of stomatal control, and on the actual rates involved, transpiration is generally less affected by low levels of soil moisture stress than is growth or metabolism, since transpiration is primarily a passive phenomenon controlled by atmospheric conditions and rate of movement of soil water to the absorbing surfaces of the roots.

The diffusion pressure deficit (DPD) (Meyer 1945) in the plant system can never fall to a value less than that of the TSMS unless there is direct absorption of water from the atmosphere by the plant leaves, since the absorption gradient from

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soil to plant is eliminated at the point of DPD-TSMS equilibrium. Thus increase in TSMS results in an associated increase in DPD in the plant, with consequent loss of turgor. It is this progressive decrease in turgor pressure, in the cells of the active tissues, which has direct and indirect effects on most plant processes.

Data from controlled experiments provide direct evidence of these effects. Reductions in rate of growth (Davis 1942; Wadleigh and Ayers 1945; Haynes 1948; Salter 1954; Gates 1955*a*, 1955*b*) and elongation (Furr and Reeve 1945; Wadleigh and Gauch 1948) have been observed to commence at low TSMS values and to become more pronounced with increasing TSMS. The effect of even small water deficits on stomatal closure (Magness, Degman, and Furr 1935; Oppenheimer and Elze 1941) is reflected in decreased rates of transpiration (Chung 1935, Martin 1940), and photosynthesis is affected through the general effects of soil moisture stress (Schneider and Childers 1941; Loustalot 1945), and through the direct effect of reduced tissue hydration (Walter 1929; Rabinowitch 1945).

In general, most studies have been concerned more with the stress value at which reductions first occurred, and with the rate of decrease with increasing stress, than with the stress value at which the particular process ceased. It has been generally assumed that growth ceases at a stress value close to that at the permanent wilting percentage (Veihmeyer and Hendrickson 1950). Slatyer (1957*a*) has suggested that permanent wilting occurs at a stress value such that there is zero turgor pressure in the leaf tissue, and that this stage would also represent a probable end-point for most of the assimilatory and developmental processes which are integrated in vegetative growth. This implies that the permanent wilting percentage is a value determined not by any particular soil characteristic but by the osmotic characteristics of the plants under study, and as a result could vary considerably from plant to plant.

The present experiment was designed to examine the response patterns shown by three different plant species to conditions of increasing soil moisture stress, and to interpret these patterns in terms of the energy relations of the plant-soil water system and the turgescence of the plant tissues. Particular attention was paid to the significance of the permanent wilting percentage as a valid indicator of the lower limit of soil water availability, and to the internal water relations of the species at very severe TSMS levels.

II. EXPERIMENTAL METHODS

(a) General

Seeds of cotton (*Gossypium barbadense* L. var. Pima S1) and tomato (*Lycopersicon esculentum* Mill. var. Marglobe) and year-old plants of privet (*Ligustrum lucidum* Ait.) were planted in prepared, fertilized soil in steel cans 8 in. in diameter and 8 in. high. Each can contained about 4.5 kg of soil and weighed about 5.5 kg when the soil was at field capacity. The cans were set out in a heated greenhouse in early winter and were grown until the plants were well established and the roots thoroughly penetrated the soil mass. Re-watering to field capacity took place when wilting of the lower leaves was evident at sunrise. This watering regime was utilized to pre-condition the plants to water stress. Each can was then given a final watering to bring the whole soil mass to a value slightly above field capacity. To prevent

direct evaporation losses, the top of each can was then covered with oilcloth and the gap around the stem was filled with cotton wool to minimize losses through this channel. The efficacy of these seals was such that on each of four control cans the total losses by direct evaporation during the final experimental period was less than 15 g. In order to minimize heating of the cans by direct sunlight, and to minimize movement of soil water in response to thermal gradients, the cans were placed on a bench which had raised sides, and the oilcloth on top of each can was itself covered with aluminium foil. The plants were then allowed to dry out the soil without further additions of water. Harvests were made 3 days before the last watering, on the day after the last watering, and at intervals of several days thereafter depending on the condition of the plants. At each harvest determinations were made of fresh and dry weight of the plant parts, stem length, leaf turgor, DPD and osmotic pressure of the leaves, and soil water contents. In order to keep the plants continuously in a vegetative phase all floral organs were removed as soon as they appeared. In addition, lateral growth in tomato was removed as soon as it appeared and the plants were trained to a single stem. All physical measurements which varied with temperature were corrected to 20°C, the basal temperature of the heated greenhouse.

(b) *Plants*

All harvests were made before sunrise on the sampling day. This procedure was adopted so that the plants had overnight to regain turgor, and to ensure comparative values from one harvest to the next. Eight replications were made of each of the rates or attributes.

At each harvest, measurements were made of the fresh and dry weights of the stems and leaves, dry weights of the roots, and of stem length. From these data total dry weights and stem lengths were used to obtain a picture of growth.

From the fresh material, 1.0-cm diameter leaf disks were punched from standard active adult leaves for determination of relative turgidity and DPD. The remainder of the leaves were wrapped in aluminium foil and frozen at -20°C for osmotic pressure determinations. After each of these determinations the appropriate fresh and dry weights were incorporated into the total dry weights for each plant. Relative turgidity was measured using Weatherley's (1950) technique. DPD was measured using a modification of the vapour equilibration method of Arcichovskij and Arcichovskaja (1931) described separately by Slatyer (1957b). Osmotic pressure determinations were made by cryoscopy on sap expressed from the thawed leaves using a hydraulic press at 15,000 lb/in² pressure. The agreement between replications in these determinations was very good, and at no harvest did the standard error of the mean values exceed ± 0.7 per cent. in the relative turgidity determinations, ± 2.3 atm in the DPD determinations, or ± 1.4 atm in the osmotic pressure determinations.

Transpiration was measured by daily weighings of all cans. The differences were corrected for the changing fresh weight of the plants, so that the data obtained represented net transfer of water from soil to atmosphere rather than total water loss from plant and soil.

(c) Soils

The soil used in this experiment was a sandy clay loam belonging to the White Store series (Perkins *et al.* 1924) of the Intrazonal Planosol group. It was selected for this experiment on the basis of prior soil moisture retention determinations which indicated that it had a fairly high soil moisture content below the value at 15 atm tension.

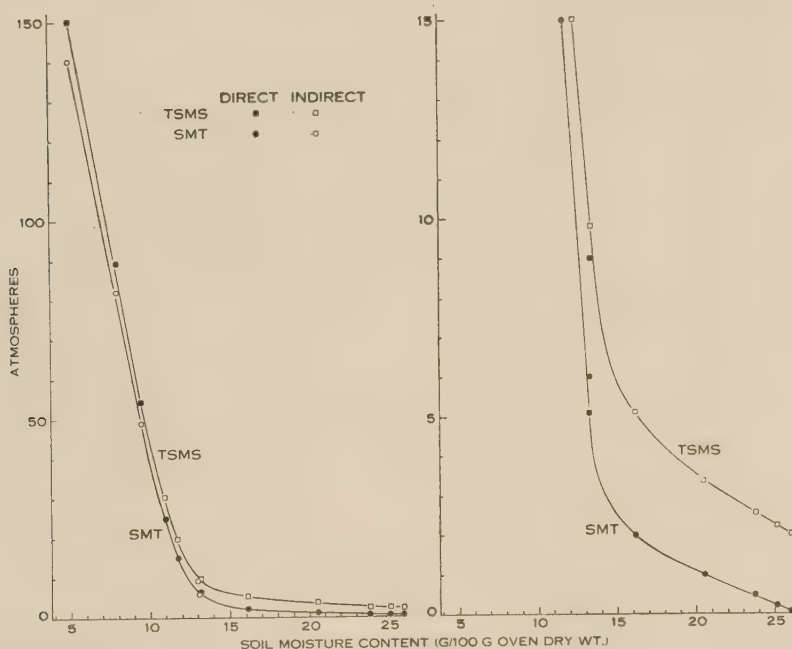


Fig. 1.—Soil water characteristic curves for the soil used in the experiment. The diagram on the right is a large-scale ($10\times$) version of part of the curve shown in the diagram on the left. The outside curve in each diagram represents the soil water content/total soil moisture stress (TSMS) relationship, the inside curve the soil water content/soil moisture tension (SMT) relationship. The key in the diagram indicates which of the points on the curves represent direct, and which indirect, determinations.

The soil was prepared by sieving three times through a $\frac{3}{8}$ in. sieve to provide as thorough mixing as possible. A complete fertilizer mixture was added during the final sieving.

At each harvest soil water content was determined by taking four cores each of $\frac{3}{4}$ in. diameter from each of eight cans. Two cores were taken near the stem and two near the edge of the can. The cores were divided into a top and bottom portion and the eight subportions were oven dried separately to obtain a gravimetric value for soil moisture content. At no stage was there any significant difference (at $P=0.01$) between the top and the bottom parts of each core or between the cores taken near the stem and those taken near the edge of the cans. This substantiated the visual indications of thorough root penetration.

Soil moisture tension values were obtained from separate undisturbed cores also taken from the cans at each harvest. Values up to 600 cm tension were obtained with a suction-plate apparatus (Richards 1942) and from 1 to 25 atm with pressure membrane equipment (Richards 1947). As it was desired to examine the reactions of the plant to total soil moisture stress rather than to soil moisture tension alone, the soil moisture tension values were adjusted to include osmotic effects. This was done in the manner suggested by Richards and Weaver (1944) who measured the osmotic pressure of the saturation extract and computed the osmotic pressure values applicable to any soil moisture content.

In addition TSMS was measured directly on soil cores by equilibrating them in vapour of known vapour pressure using a modification of the method of Thomas (1921) and Edlefsen (1934). The modification involved the use of micro-desiccators and very accurate temperature control, both of which improved the precision of the determinations. The direct determinations made in this manner were in good agreement with the indirect estimates which combined soil moisture tension and osmotic pressure determinations. In Figure 1 data for soil moisture tension and TSMS are plotted against soil moisture content. Soil moisture tension levels in excess of 25 atm were computed by adjusting the direct TSMS measurements for osmotic pressure.

Each point on these curves was made up from at least 24 separate determinations, and at 200 cm, 15 atm, and 150 atm 64 determinations were made. The standard error of the mean value of any one set of determinations did not exceed ± 0.16 per cent. soil moisture content.

On each sampling day, the soil moisture content was obtained from gravimetric determinations on the cores as described above, the mean value for any one harvest being obtained from 32 subsamples. TSMS and soil moisture tension values equivalent to the soil moisture contents were obtained from the subsequently derived calibration curves.

There was also a high order of accuracy in these determinations, the standard error of the mean at any one sampling occasion not exceeding ± 0.15 per cent. for any species. This variation was equivalent to not more than ± 3.2 atm TSMS even at high stress values.

(d) Atmospheric Conditions

A white atmometer and thermohygrograph were installed in the greenhouse on the same bench as the cans. This enabled estimates of evaporation, temperature, and relative humidity to be obtained. During the sampling period the daily maximum temperatures did not fluctuate beyond the range of 86–97°F, except for a cloudy period at the end of the experiment when temperatures fell to 80°F. On sunny days the greenhouse was opened as soon as the temperature approached 90°F. The minimum temperatures were held to the basal temperature of the heated greenhouse 65°F. Evaporation from the white atmometer ranged from 16–24 c.c. per day. Supplemental light was provided to give a 15-hr day to all plants.

III. RESULTS

Primary data from the three crops is presented in Figure 2. The first harvest shown on the diagrams was that made on the morning after the final watering. The date of the first harvest of privet was 2 days after tomato, and of cotton was 3 days after privet. This was done in order to avoid, where possible, two harvests on the one day, which presented considerable handling difficulty. Because the climatic conditions did not vary much over the experimental period the variation introduced by this procedure was slight.

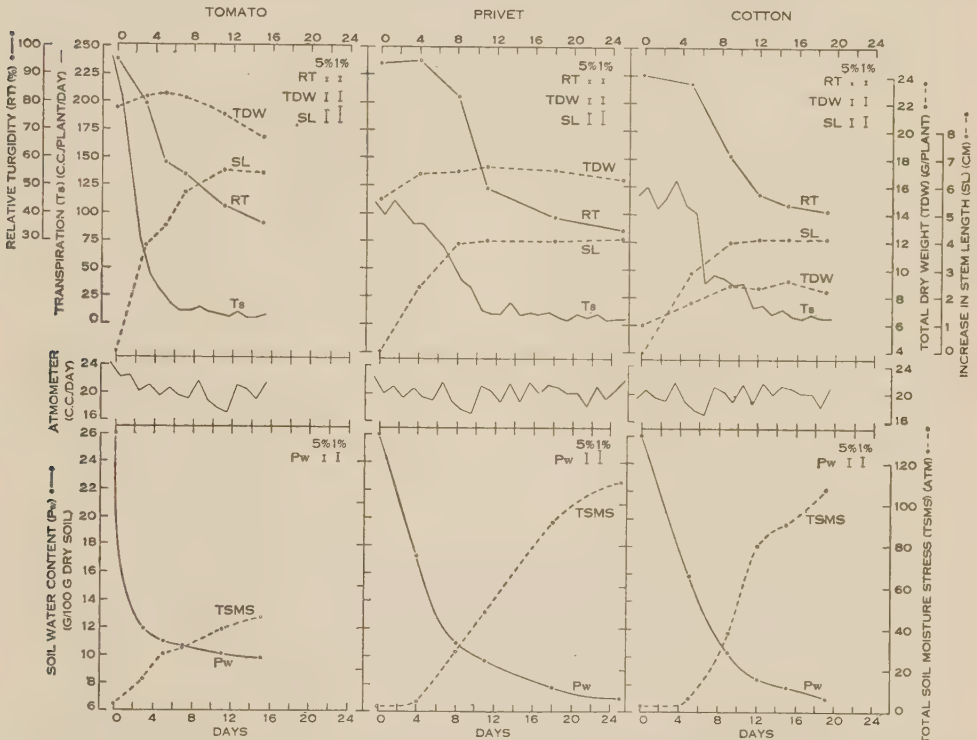


Fig. 2.—Growth (TDW), increase in stem length (SL), relative turgidity (RT), and transpiration (Ts) of tomato, privet, and cotton, in relation to soil water content (Pw) and total soil moisture stress (TSMS). Daily atmometer evaporation is shown in the body of the diagram. When applicable, minimum significant differences at 5 per cent. and 1 per cent. levels are indicated.

The tomato plants were by far the largest at the beginning of the experimental period. As a result, transpiration per plant was initially much higher, and rapid soil moisture depletion caused an early onset of water stress in these plants. The first evidence of water stress in tomato was a sharp drop in transpiration after 2 days, and an associated progressive decrease in relative turgidity. Water extraction continued so rapidly that on day 3 a soil moisture stress value of 15 atm was observed. At this time relative turgidity had fallen to 78 per cent. and transpiration was at about one-third of the maximum rate. No significant increase in dry weight was observed after this time. Transpiration continued to decrease rapidly until, at a

TSMS of about 30 atm, it reached a level of less than 10 g/plant/day, at which it remained fairly stable to the end of the experimental period. This rate was about 5 per cent. of the maximum rate. Leaf turgor levels continued to fall, but stem elongation continued until approximately 38 atm soil moisture stress existed. The last harvest was made when the lower leaves were dying, and when the first dead patches were appearing on the leaves used for water relations purposes. On this occasion the soil moisture stress was 45 atm, and the reduction in soil water content below the 15 atm value was 2.4 per cent. The first visible signs of wilting were observed

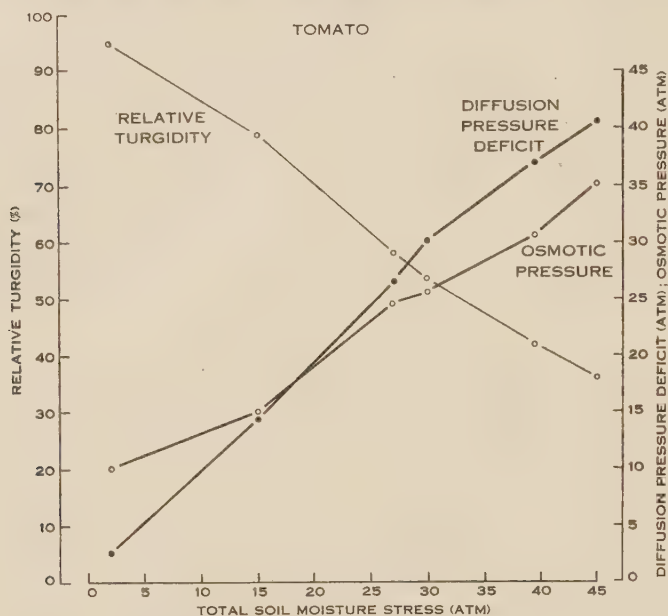


Fig. 3.—Diffusion pressure deficit, osmotic pressure, and relative turgidity of adult leaves of tomato in relation to increasing total soil moisture stress.

on day 2 when diurnal wilting around midday was apparent. At dawn on day 4, the lower leaves appeared completely wilted and this appearance was regarded as providing an approximate indication of the first permanent wilting percentage of Furr and Reeve (1945). At this stage the soil moisture stress was 20 atm and the relative turgidity in the upper leaves was 70 per cent. Stem elongation, frequently a good indicator of the first permanent wilting percentage, continued beyond this point although at a reduced rate, and transpiration was at a level of about 15–20 g/day. At dawn on day 7 the apical leaves appeared severely wilted and this was considered to indicate the approximate ultimate permanent wilting percentage. By then some of the lower leaves were dying, and total dry weight was decreasing. The soil moisture stress at this stage was 32 atm.

Evidence concerning the physical response of the tomato to increasing stress is provided in Figure 3 where the relative turgidity, DPD, and osmotic pressure of the leaf tissue is plotted against TSMS. Although the sample leaves used for these deter-

minations were in the upper part of the plants, their relative turgidity/DPD relationships resembled those of the lower leaves rather than of the apical leaves, as the majority of the leaves wilted at about the same TSMS, and only the apical leaves exhibited delayed wilting. This also applied in cotton and privet, except that in privet the previous season's leaves were so rigid as to not exhibit any signs of wilting. In this case only the current season's growth could be used as an indicator.

From Figure 3, it is evident that the DPD values at dawn were generally in good agreement with the TSMS, indicating that turgor recovery normally proceeded overnight to a stage equal to DPD-TSMS equilibrium. As TSMS increased further it could be expected that, because of slower recovery of turgor, this equilibrium value would not be reached by dawn, so that the DPD would remain in excess of the TSMS. Examination of Figure 3, however, shows that on the last two harvests the DPD values were actually lower than the TSMS. This could have arisen from direct nocturnal absorption of water vapour from the atmosphere by the leaves of the plants, and the possibility of this occurring is considered in the discussion.

The changes in osmotic pressure which followed the onset of stress were slightly more than could be expected as a result of simple concentration of the cell sap following tissue dehydration. This is probably a result of breakdown of some organic complexes to osmotically active substances which frequently occurs as stress is imposed (Meyer 1956). An interesting feature of the osmotic pressure curve was that the osmotic pressure became less than the DPD at high stress levels. On the day of the last harvest this difference was 5 atm. If the classical osmotic theory holds, the osmotic pressure should never be less than the DPD unless other sources of negative pressure are being developed. The possibility of this occurring will also be considered in the discussion.

In privet the smaller leaf area of the plants resulted in less rapid transpiration than in tomato, and hence slower soil moisture depletion. The first sign of stress was a cessation of significant increases in total dry weight on day 4, when 5 atm soil moisture stress existed. No significant decrease in leaf turgor was evident at this stage, and reduction in transpiration rate was only just commencing. Subsequently a progressive and steady decrease in turgor and transpiration occurred. Stem elongation proceeded at a reduced rate until it ceased when the relative turgidity was 82 per cent. and the soil moisture stress 29 atm. First evidence of permanent wilting occurred at 49 per cent. relative turgidity, a much lower value than in tomato, and ultimate wilting occurred soon afterwards at a relative turgidity of 46 per cent. The appropriate TSMS values were 48 and 60 atm.

Transpiration declined to a low and fairly stable level of about 5-8 g/plant/day at about the time first wilting was observed, and continued at this rate until the end of the experiment. The final harvest was made on day 25 when, as in tomato, dead patches appeared on the sample leaves. At this last harvest the relative turgidity was 34 per cent. and the TSMS 110 atm. This value represented a decrease of 5.3 per cent. soil moisture content below the value observed at 15 atm TSMS.

In Figure 4, the response of privet in terms of leaf turgor, DPD, and osmotic pressure is shown in relation to TSMS. The DPD at zero turgor pressure was 27 atm,

and the relative turgidity 83 per cent. Stem elongation ceased at an almost identical value. The association of the state of zero turgor pressure with first permanent wilting, evident in tomato, was not applicable in the case of privet, and appeared to be due primarily to the rigid leaf characteristics of privet, which tended to defer the appearance of wilting until high stress values obtained.

The responses of privet in terms of DPD and osmotic pressure were of particular interest. As in tomato, there was good agreement between the dawn values of TSMS and DPD in the early stages of dehydration. Beyond about 50 atm stress,

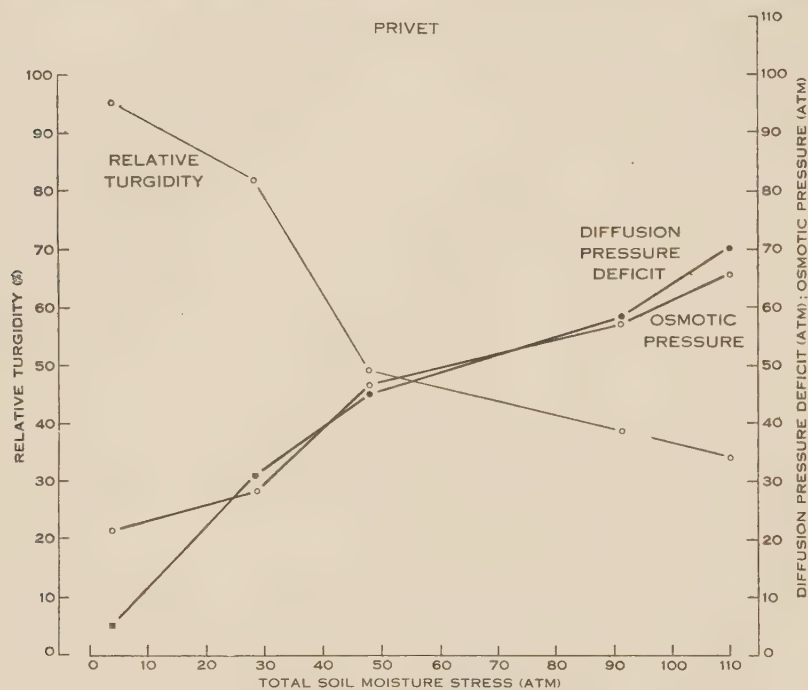


Fig. 4.—Diffusion pressure deficit, osmotic pressure, and relative turgidity of adult leaves of privet in relation to increasing total soil moisture stress.

however, the DPD values fell below those of TSMS and the divergences became progressively more marked so that, at the last harvest, the DPD was 70 atm and the soil moisture stress 110 atm. This provided further strong evidence for the occurrence of direct absorption from the atmosphere. The response of osmotic pressure was similar to that in tomato, and again the change in osmotic pressure with increasing stress was only slightly more than would be anticipated due to increase in cell sap concentration due to dehydration alone. Whereas in tomato the osmotic pressure fell below the DPD at high stress levels, in privet there was good agreement of DPD and osmotic pressure at DPD's greater than those at zero turgor pressure.

In cotton the first evident response to stress was a reduction in relative turgidity. Transpiration showed little change until 10 atm stress, when it decreased in the space of 2 days from a value of the order of 100 g/plant/day to 35 g/plant/day.

A marked reduction in rate of stem elongation occurred at the same time, which corresponded to a relative turgidity of 83 per cent. Increase in dry matter and elongation continued until relative turgidity had fallen to 62 per cent. and TSMS was 38 atm. This corresponded closely with first appearance of permanent wilting.

Transpiration rate continued to fall after this stage until it reached a fairly steady rate of 6-8 g/plant/day at about 60 atm TSMS. At the conclusion of the experiment, relative turgidity was 41 per cent. and TSMS 107 atm. This represented a reduction of 5.2 per cent. soil moisture content below the percentage at 15 atm TSMS.

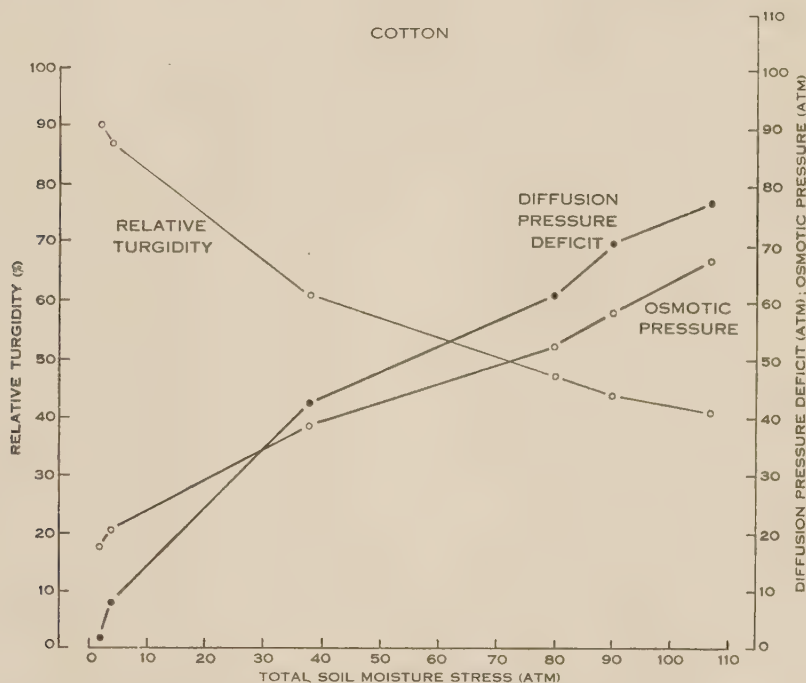


Fig. 5.—Diffusion pressure deficit, osmotic pressure, and relative turgidity of adult leaves of cotton in relation to increasing total soil moisture stress.

From Figure 5 it can be seen that the DPD at zero turgor pressure, 34 atm, corresponded to a relative turgidity of 66 per cent. This is in good agreement with the cessation of growth and elongation, and with the appearance of permanent wilting. Ultimate wilting occurred 1 day after first permanent wilting at a TSMS of 50 atm.

The responses of cotton in terms of DPD and osmotic pressure show characteristics of both the privet and tomato patterns. The strong divergence of DPD and TSMS at high stress levels observed in privet was again evident, suggesting, as before, direct vapour absorption by the leaves. The change in osmotic pressure was again only slightly more than could be expected from the direct effect of dehydration on sap concentration. Also of interest was the point observed earlier in tomato, that the osmotic pressure fell below the DPD at DPD's higher than those

at zero turgor pressure. The fact that good agreement occurred in privet would appear to be evidence for the validity of the measurement techniques and it would seem that negative pressure from sources other than of a direct osmotic nature was contributing to the DPD at high tensions in the other two species.

IV. DISCUSSION

Water stress has a marked effect on all plant processes and it is to be expected that this would be more pronounced on active processes which have their expression in elongation or vegetative growth than on predominantly passive processes such as transpiration. As soil moisture stress increases, turgescence must decrease, and the degree of turgidity which can be maintained by a plant is limited by the TSMS, as the absorption gradient is eliminated at the point of DPD-TSMS equilibrium. Thus, nocturnal recovery in turgor by a plant ceases when a state of DPD-TSMS equilibrium exists in the soil-plant system, unless additional water is absorbed directly from the atmosphere by aerial plant organs.

The progressive decrease in plant turgor which accompanies increase in soil moisture stress usually causes a progressive decrease in growth and elongation, as demonstrated by the detailed studies of Davis (1942), Furr and Reeve (1945), Scofield (1945), Wadleigh and Ayers (1945), Haynes (1948), Wadleigh and Gauch (1948), Bernstein and Pearson (1954), and Gates (1955*a*, 1955*b*). Decrease in transpiration is also to be expected because of the effect of turgescence on stomatal closure, and because of the slower rates of soil water movement to the absorbing surfaces of the roots. Growth (as increase in total dry weight) and elongation will finally be completely inhibited by decreasing hydration, and it is logical to assume that in any one tissue growth will cease at or before a DPD exists such that there is zero turgor pressure in the cells of the tissue concerned. Such a degree of dehydration should be reflected in a permanently wilted appearance of the leaves, and Slatyer (1957*a*) has suggested that this point corresponds to the point at which the permanent wilting percentage occurs.

In the results presented above this hypothesis appears valid. In tomato and cotton, increases in dry matter ceased approximately at the same DPD as that at zero turgor pressure in typical adult leaves. In privet, cessation occurred at much lower stress values. This may have been due partly to sampling error and partly to the fact that the relative turgidity at zero turgor pressure was highest in privet. This would mean that the relative cell volume at full turgor would be lowest in this species, so a slight reduction in turgidity would cause a proportionately greater reduction in cell volume and turgor pressure than in the other crops, which may in turn have caused inhibition of growth at lower stress values.

Cessation of elongation in privet and cotton occurred at stress values close to those obtaining at zero turgor pressure in the adult leaves. In tomato, elongation continued until very severe stress existed, and until the plant as a whole was losing dry weight. A similar result was reported for tomato by Wilson (1948). It is probable that in young elongating tissue, most of the cells are not completely vacuolated and osmotic theory is not strictly valid. Kerr and Anderson (1944) considered that imbibitional forces in developing cotton bolls enabled development to proceed at

water deficits which stopped the growth of other tissues, and a similar situation possibly existed in the apical tissue of tomato.

Because transpiration is primarily a passive phenomenon it does not necessarily cease at any particular stress level. It could be expected, however, that the transpiration rate would be reduced at low stress levels because of stomatal closure and reduced rates of soil water movement in unsaturated soils. Such reductions have been observed, for instance, by Chung (1935), Martin (1940), and Slatyer (1956*a*). Subsequently there seems to be no reason why transpiration should not continue until, or beyond, the death of the plant, limited only by the energy available for evaporation, the resistance to water movement into, through, and out of the plant, and by the rate of flow of soil water to the roots.

Although Veihmeyer and Hendrickson (1950, 1955) have claimed that transpiration is unaffected by TSMS until the permanent wilting percentage is reached when, in field soils, they consider that it virtually ceases, there is no valid physical or physiological reason why this should necessarily be so (Slatyer 1957*a*), and in general investigations of plants in their natural habitats (Alway 1913; Burr 1914; Batchelor and Reed 1923) and in containers (Briggs and Shantz 1912; Hendrickson and Veihmeyer 1945) have demonstrated continued absorption of soil water to values well below the permanent wilting percentage. In the present study these general contentions were borne out, reductions in transpiration rate first occurring at low stress values, but continued transpiration being observed, although at reduced rates, until the conclusion of the experiment, when the soil water content was 9.8, 6.9, and 7.0 per cent. for tomato, privet, and cotton respectively. This represented a decrease of 2.4, 5.3, and 5.2 per cent. respectively below the value at 15 atm TSMS.

The fact that privet and cotton continued to extract soil water at TSMS values beyond those which caused the death of tomato, appeared to be due to the DPD/relative turgidity characteristics of the different plants. Death no doubt ensues once a certain degree of dehydration has been reached, and the DPD appropriate to this critical value is probably reflected in the TSMS level at the death point. Marked differences in the DPD/relative turgidity characteristics of different plants exist (Weatherley and Slatyer 1957) and it is evident from Figures 3-5 that at similar stress values, relative turgidity was much lower in tomato than in the other species.

The significance of the permanent wilting percentage in studies of plant and soil water relations must be reduced when it is recognized that the permanent wilting percentage is not a soil constant as suggested by Briggs and Shantz (1912) and Veihmeyer and Hendrickson (1927, 1950) but is a value dependent primarily on the osmotic characteristics of the plants under study. Slatyer (1957*a*) proposed that permanent wilting occurred when the DPD reached a value such that there was zero turgor pressure in the leaves of the plant under examination and that the permanent wilting percentage was consequently at the TSMS value which corresponded to this DPD. This would be reflected in wilting of the leaves and the approximate cessation of growth.

In the present study the DPD at zero turgor pressure in the sampled leaves was 17, 27, and 34 atm in tomato, privet, and cotton respectively. Beyond this degree of stress there was no further growth or elongation except in the case of tomato, where elongation continued until severe stress existed. This meant that the concept of permanent wilting being associated with growth cessation was valid for any one species, but it also demonstrated that no one TSMS value, and hence no single soil constant, could be ascribed to the stage of permanent wilting of all species, as the wilting of any plant is a function of its turgor pressure, which at any one DPD or TSMS level is determined by its osmotic pressure. The order of variation between the three species examined in the present study, with respect to osmotic pressure, DPD, TSMS, and soil water content at first permanent wilting, and at the conclusion of the experiment, is shown in Table 1.

TABLE 1

OSMOTIC PRESSURE, DIFFUSION PRESSURE DEFICIT (DPD), TOTAL SOIL MOISTURE STRESS (TSMS), AND SOIL WATER CONTENT AT FIRST PERMANENT WILTING PERCENTAGE AND AT THE CONCLUSION OF THE EXPERIMENT

Plant	First Permanent Wilting Percentage*				Conclusion of Experiment			
	Osmotic Pressure (atm)	DPD (atm)	TSMS (atm)	Soil Water Content (%)	Osmotic Pressure (atm)	DPD (atm)	TSMS (atm)	Soil Water Content (%)
Tomato	18	19	20	11.8	35	41	45	9.8
Privet	47	45	48	9.7	66	70	110	6.9
Cotton	38	43	38	10.2	67	77	107	7.0

*Using the terminology of Furr and Reeve (1945). For comparative purposes the soil water content at 15 atm TSMS was 12.2 per cent.

Errors can be introduced in any wilting experiment because of the difficulties in recognition of wilting in rigid leaves. In tomato and cotton the estimated TSMS at which first permanent wilting occurred bore a close relationship to the DPD which obtained at zero turgor pressure (tomato, 20 : 17; cotton, 38 : 34). In privet, on the other hand, wilting was not evident at dawn until 48 atm TSMS existed (cf. DPD of 27 atm at zero turgor) because of the rigid characteristics of the leaf tissue. The soil water content at the first permanent wilting percentage was 11.8, 9.7, and 10.2 per cent. for tomato, privet, and cotton respectively. The last two values differed markedly from the soil water content at 15 atm TSMS, the value usually ascribed to the first permanent wilting percentage (Richards and Weaver 1944; Veihmeyer and Hendrickson 1950), which was 12.2 per cent.

The estimates of wilting were made by accepting a strongly wilted appearance at dawn as the wilting index. Such an index could introduce errors if severe water deficits had been developed on the preceding day, and the overnight period had not

been of sufficient duration to enable DPD-TSMS equilibrium to be established by sunrise. Reference to Figures 3-5 demonstrates that at the stress values which obtained at first permanent wilting percentage, there was DPD-TSMS equilibrium at dawn, and so the acceptance of a dawn appearance as an indication of the first permanent wilting percentage seemed to be valid. At ultimate wilting there was evidence in privet and cotton that the DPD values at dawn were less than the TSMS, and the use of the index in this connection is not as sound.

The responses of the plants to increasing soil moisture stress, in terms of DPD, osmotic pressure, and relative turgidity of the leaves, are of particular interest. As soil moisture stress increased, the changes in relative turgidity were reflected by opposite changes in DPD. The relationship between these responses is of considerable practical significance as the degree of turgidity at any one stress value is indicative of the physiological activity of the plant. Increase in osmotic pressure as soil moisture stress increased was only slightly more than was to be expected as a direct result of dehydration on solute concentration. It would appear that in each species the onset of stress was too rapid for the significant development of other sources of osmotic pressure by the breakdown of organic complexes to osmotically active substances, as was suggested by Ahrns (1924) and Spoehr and Milner (1939).

As dehydration proceeds beyond the state such that there is zero turgor pressure in the cells of a tissue, Meyer (1956) suggested that the water in the cell passes into a state of tension and the cell walls are subject to a strong centripetally directed pull due to the strong adhesion between the protoplast and the cell wall. This causes the development of negative turgor pressure and Thoday (1921) and Engmann (1934) have observed inward folding of cell walls as a result of such pressure during severe wilting. It would seem that the magnitude of the negative pressure which can develop in this manner must be limited by the degree of adhesion between the protoplast and the cell walls. Buhmann (1935) found values up to 10 atm for some species and reported similar results of earlier workers. In the present experiment little evidence of this effect was noted in privet, even under extreme stress, but in tomato the DPD exceeded the osmotic pressure by up to 5 atm, and in cotton the differences were as high as 10 atm.

As mentioned earlier, it is to be expected that as TSMS increases DPD must also increase, as the DPD cannot fall to a value lower than the TSMS unless an absorption gradient develops from atmosphere to plant and water is directly absorbed by the shoots. Even if absorption occurred in this manner, water should not accumulate in the plant, for the gradient should exist not only from atmosphere to plant, but from atmosphere to plant to soil, and movement ("negative transport") should continue into the medium surrounding the roots (Breazeale, McGeorge, and Breazeale 1950, 1951; Breazeale and McGeorge 1953*a*, 1953*b*; Haines 1952, 1953; Slatyer 1956*b*). For these reasons it was surprising to find, in the present experiment, evidence of water accumulation in the plants against a plant-soil diffusion gradient. In Figures 3-5 it can be seen that in privet and cotton there was a marked reduction in the DPD below the TSMS as TSMS exceeded a level of about 50 atm, and that in tomato, reductions were evident at TSMS levels of 30 atm and over. Although the reductions

in DPD were considerably less in tomato than in the other species, the amounts of water needed to cause the observed reductions were of the same general order in all species due to the much greater total weight of the tomato plants.

As it was not anticipated that evidence of absorption would be found, night and morning weights of cans were not taken to confirm that absorption definitely took place. However, there seems no doubt that the reductions were real, for TSMS was measured to within ± 3 atm and the validity of the DPD determinations was demonstrated by the good agreement between TSMS and DPD at low stress values, and the parallelism between the DPD and osmotic pressure curves at high stress values. Furthermore, by utilizing greenhouse temperature and humidity records it was computed that on each of the nights preceding harvests the amount of water in the atmosphere directly over each plant, which would have been yielded as dew, was always greater than the amount needed to cause the observed reductions in DPD in the plants.

A possible explanation for these results is that at low stress values, negative transport occurred, water being absorbed by the top of the plant and moving through the plant, along a gradient of increasing DPD, into the soil. As stress increased, suberization and death of roots, together with tissue and soil shrinkage, caused a progressive decrease in continuity in the water system between soil and root, so that the diffusion paths were progressively eliminated, and water tended to accumulate more and more in the plant. This hypothesis is supported by the evidence that transpiration decreased with increasing stress, until it reached a low and stable level at high stress values. These values corresponded very closely to those at which evidence of accumulation first appeared and the same explanation of discontinuity in the root-soil system could be applied to both phenomena. It also offers an explanation as to why, in separate experiments, Breazeale *et al.* (loc. cit.) were able to demonstrate negative transport of simulated dew through plants and into moist soil, but Stone, Shachori, and Stanley (1956) were unable to demonstrate negative transport into very dry soil, even though the tops of their plants regained turgor.

This has an interesting practical implication, as it suggests a mechanism whereby, under natural conditions, plants in dry soil could use atmospheric water for re-saturation of tissues without losing this water to the soil. Although absorption could theoretically occur whenever a favourable gradient existed there would be no real physiological benefit unless positive turgor pressure could be re-established in the active tissues. Thus the DPD of the water in the atmosphere would have to be low enough to reduce the plant DPD to a level below that at zero turgor pressure. If this were to occur, it is possible that sufficient photosynthesis could proceed in early morning, before transpiration re-established severe water deficits, to enable the plant to balance its respiratory losses. In this case a plant could conceivably persist for extended periods without further re-charge of soil water.

V. ACKNOWLEDGMENT

This study was conducted at Duke University, Durham, North Carolina, in the laboratory of Dr. Paul J. Kramer to whom grateful acknowledgment is made for the provision of research facilities, financial assistance, and valued discussion.

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THE EFFECT OF 3-INDOLYLACETIC ACID ON THE BINDING OF PECTIN METHYLESTERASE TO THE CELL WALLS OF TOBACCO PITH

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[Manuscript received March 20, 1957]

Summary

3-Indolylacetic acid (IAA) was found to promote the binding of pectin methylesterase (PME) to a cell wall fraction prepared from tobacco pith, the effect being increased by increasing concentration of IAA to a peak of activity, after which inhibition occurred.

It is suggested that the immobilization of PME, by binding to the cell wall *in vivo*, would favour methylation of the pectates of the cell wall and hence increase cell wall plasticity.

I. INTRODUCTION

The multiplicity of physiological effects of 3-indolylacetic acid (IAA) on the growth of plants has served to develop the view that the primary site of action of the hormone is of very great importance in cellular metabolism. Many investigators have considered that this reaction is closely associated with the pectic substances of the cell walls (van Overbeek 1952; Bennet-Clark 1955), and that the control of cell wall plasticity constitutes the major effect of IAA in the cell (Burstrom 1953; Levitt 1953).

McRae, Foster, and Bonner (1953), in studying the kinetics of auxin effects on the growth of the oat coleoptile, concluded that each auxin molecule was bound at two points to a cellular component to form a complex active in stimulating growth. At high levels of auxin concentration, a separate molecule was bound at each point causing inhibition of growth. Ordin, Cleland, and Bonner (1955) have shown that the incorporation of methionine-¹⁴C, labelled in the methyl group, into the cell wall constituents of oat coleoptiles was enhanced by auxin, and that enhancement of incorporation into pectins occurred even if the growth of the tissues was suppressed by suitable external osmotic concentrations.

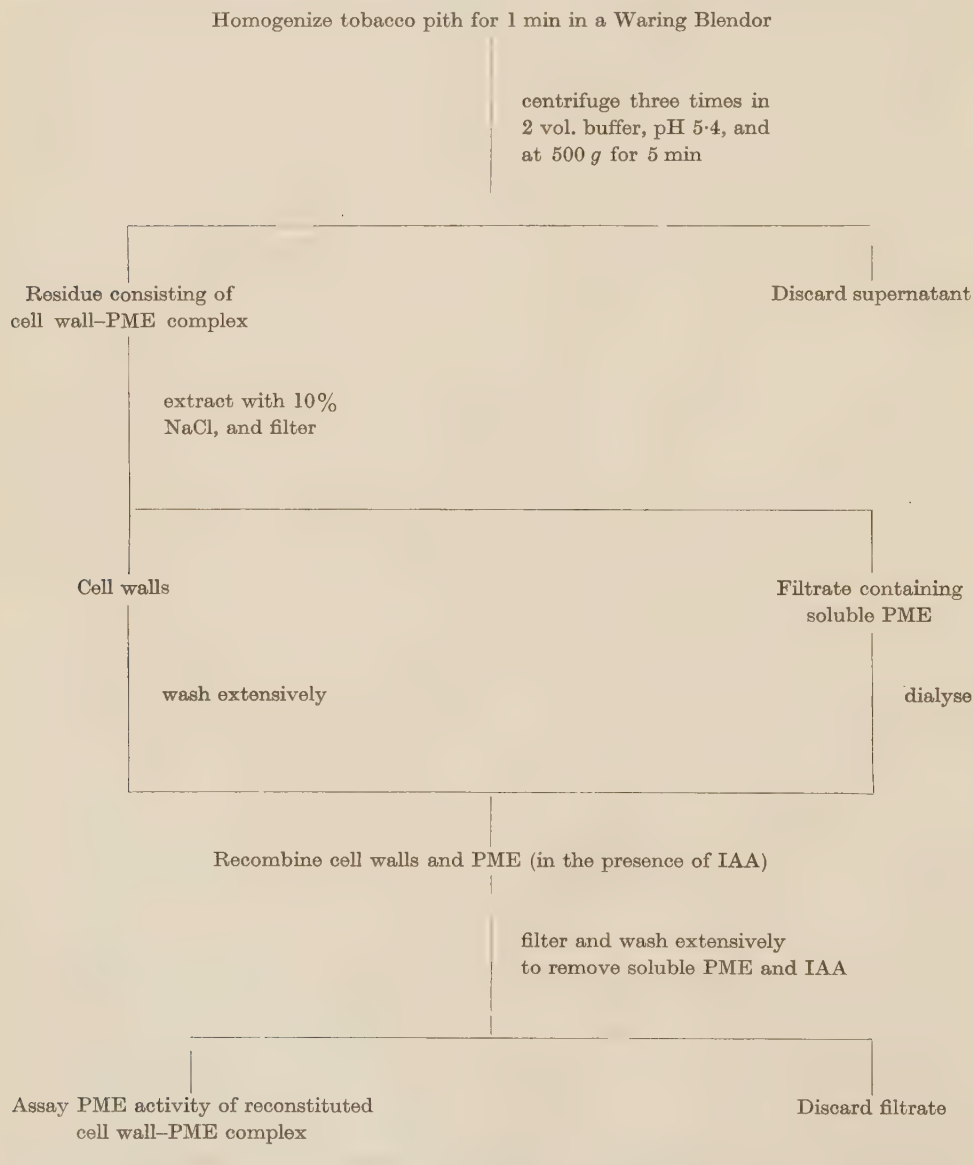
Wilson and Skoog (1954) studied the uronide composition of tobacco pith sections which had been stimulated into rapid cell enlargement by IAA, and observed that the changes in uronide and pectic materials which resulted from IAA treatment could be considered as an integral part of auxin-induced growth of cell walls. Bryan and Newcomb (1954) also studying the growth of tobacco pith sections observed that IAA-induced cell enlargement was accompanied by an increase in pectin methylesterase (PME) activity to almost twice the value for the control sections after 120 hr. These authors found that in a cell wall fraction which contained almost all the PME activity of the tissue, the enzyme activity was reduced over a wide range of concentrations by IAA.

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It is pertinent to consider whether IAA functions by promoting the binding of enzymes of cell wall metabolism to loci within the cell. The results presented indicate

TABLE 1

FLOW DIAGRAM OF METHOD OF CLEAVING AND RECONSTITUTING THE CELL WALL-PECTIN METHYLESTERASE (PME) COMPLEX FROM TOBACCO PITH (SEE TEXT FOR FULL DETAILS)



that IAA promotes the binding of one such enzyme (PME) to constituents of the cell wall. To demonstrate this, the enzyme was split from a cell wall preparation and the effect of IAA on the reconstitution of an enzyme-wall complex studied.

II. EXPERIMENTAL

A flow diagram outlining the general method of preparation of the cell wall-enzyme complex is shown in Table 1, and the procedure for one experiment is given.

Tobacco pith (28 g fresh weight), from the stems of large tobacco plants which had flowered, was homogenized in a Waring Blendor for 1 min with 60 ml 0.05M phosphate buffer at pH 5.4. The homogenate was centrifuged at 500 *g* for 5 min and the supernatant discarded. The centrifugation was repeated twice more after resuspension of the residue in 50 ml of the same buffer. To the residue was added 15 ml 0.01M phosphate buffer, pH 7.6, the volume was measured (36 ml), and the appropriate amount of NaCl added (3.6 g). The suspension was stirred at intervals over a 1-hr period and then filtered through Whatman No. 5 filter paper on a Buchner funnel. The filtrate (containing the PME) was transferred to a dialysis sac and dialysed against 5 l. distilled water with rapid mechanical stirring for 4 hr. The dialysate (PME solution) had a final volume of 41 ml.

TABLE 2
EFFECT OF 3-INDOLYLACETIC ACID (IAA) ON THE BINDING OF PECTIN
METHYLESTERASE (PME) TO THE CELL WALL

The complete system contained 1.5 g wall fraction, 7.0 ml enzyme solution, and IAA at the concn. shown. Final vol. 9.5 ml. After 90 min incubation at 25°C, systems containing the cell wall fraction were filtered, washed extensively, and the PME activity of the residue determined by measuring the acid formed during the hydrolysis of methyl ester groups of pectin

System Components	Ml 0.01N NaOH Required to Neutralize the Acid Produced in 30 Min
No enzyme; no IAA	2.0
No IAA	4.0
Complete (1.05×10^{-8} M IAA)	6.0
Complete (1.05×10^{-10} M IAA)	7.2
Complete (1.05×10^{-12} M IAA)	5.3
No wall fraction; no IAA	7.9

To obtain a cell wall fraction with low PME activity, the residue was extracted on a Buchner funnel (Whatman No. 4 paper) with 10 per cent. NaCl buffered at pH 7.2 with 0.01M phosphate buffer. Suction was applied periodically and more NaCl solution added, a total of 100 ml being used over a 3-hr period. The filtrate from this extraction contained residual PME and was discarded. The wall fraction was then washed twice with 0.05M phosphate buffer, pH 5.4, most of the buffer solution removed by suction, and the residue divided into five 1.5-g portions. All operations to this point were carried out in a cold room at 2-4°C.

Six systems were prepared, the complete system containing 1.5 g wall fraction, 7.0 ml PME solution, and IAA at the concentrations shown in Table 2 in a final volume of 9.5 ml. The pH of the incubation mixture was about 5.4, due to the phosphate buffer carried over in the wall fraction.

After 90 min incubation at room temperature (25°C) each system containing the wall fraction was filtered on a Buchner funnel with suction (Whatman No. 4 paper) and washed with 150 ml 0.001M phosphate buffer, pH 5.4, added in portions of 15 ml. After the last washing, the wall fraction was dried by suction and then transferred, complete with filter paper, to the PME assay mixture, and stirred with a glass rod to separate the wall fraction from the paper (which was then removed). System 6 (see Table 2) was added without filtering.

Enzyme assay.—The method used was essentially that described by Bryan and Newcomb (1954) except that the pH of the assay solution was adjusted not to 7.0 but to 7.5 (glass electrode) before and after the addition of the enzyme.

III. RESULTS

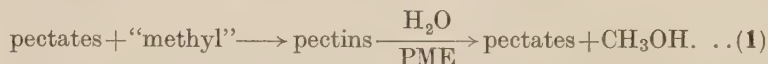
The results presented in Table 2 are typical of those obtained in a series of experiments and show that IAA is effective in promoting the binding of PME and the cell wall fraction in such a way that the enzyme cannot readily be washed off with 0.001M phosphate buffer, pH 5.4, the optimal effect being found at an IAA concentration of 1.05×10^{-10} M. The peak has been noted at 1×10^{-10} M IAA where the range extended from 10^{-6} to 10^{-12} M. However, preliminary experiments in which tobacco pith was taken from the third to the ninth internodes of young growing plants indicate that the optimal concentration of IAA is much higher in this tissue, being of the order of 1×10^{-6} M.

The possibility that the filter paper, which was used during the washing procedure following the recombining of the wall fraction and the enzyme, had affected the results by absorbing some of the enzyme was eliminated by carrying out experiments in which filter paper replaced the wall fraction. The negligible adsorption of PME to the filter paper, either in the presence or absence of IAA, may indicate that the cellulose fibres in the cell wall are not a point of attachment for PME. The effect of IAA on the activity of soluble PME was also examined, no differences from controls being observed when IAA was present in the assay system at final concentrations of 1×10^{-4} to 1.6×10^{-7} M. Similarly the effect of IAA on the activity of PME when bound to the wall was examined. No inhibition was observed even at concentrations as high as 8×10^{-4} M when added to the assay systems. This latter result is at variance with those of Bryan and Newcomb (1954) who observed reduced activity over a wide range of concentrations and complete inhibition at 1×10^{-4} M.

IV. DISCUSSION

It is well established that PME of higher plants is usually strongly adsorbed to the water-insoluble components (Willaman and Hills 1944, quoted by Kertesz 1951), and in tobacco pith it is almost completely in the cell wall fraction (Bryan and Newcomb 1954). The enzyme is readily desorbed by treatment with salt solutions, suggesting that the bonding between enzyme and wall is of a salt-like nature. The evidence presented here indicates that IAA participates in the bonding of PME to the cell wall. This concept would explain more satisfactorily the auxin effects shown by many synthetic compounds than the hypothesis that such compounds act as the prosthetic group of an enzyme.

It appears possible that the effect of IAA on the binding of PME to the cell wall would lower the activity of the enzyme in the cell, and decrease the rate of hydrolysis of pectins, which would bring about a shift in the balance of the reactions shown in equation (1) favouring accumulation of pectins:



Such a hypothesis is in accordance with current concepts that methylation of pectates increases cell wall extensibility (Bennet-Clark 1955) and results in plastic extension of the wall under the influence of the turgor pressure of the cell contents.

Similar results have been obtained with 2, 4-dichlorophenoxyacetic acid and will be the subject of a subsequent paper.

V. ACKNOWLEDGMENTS

The work described in this paper was undertaken as part of a joint research programme of the Colonial Sugar Refining Co. Ltd., the Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O., and the Botany School, University of Sydney. The author wishes to thank the management of the Company for permission to publish this paper, Dr. R. N. Robertson for his interest and advice throughout the course of the work, and Professor Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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THE METABOLIC FATE OF TRIS-1,10-PHENANTHROLINE ¹⁰⁶RUTHENIUM (II) PERCHLORATE, A COMPOUND WITH ANTICHOLINESTERASE AND CURARE-LIKE ACTIVITY

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[Manuscript received October 22, 1956]

Summary

Tris-1,10-phenanthroline ruthenium(II) perchlorate is a representative of a group of stable inorganic complex compounds with anticholinesterase and curare-like activities.

The labelled ruthenium complex has been prepared and resolved into its optical antipodes. The metabolic fate of the stereoisomers and the racemate was investigated.

The complex administered intraperitoneally was not metabolized by the tissues of mice and rats. It was excreted mainly in the urine. No traces of it could be found in nervous tissues. It accumulated appreciably in kidney and liver but the amounts found in other tissues were below that in blood.

After intraperitoneal administration the (+) and (±) forms of the complex appeared at a greater rate in blood and urine than the (−) form.

Atropine, which has been found to enhance the toxicity of the complex, raised the rate of appearance of the (−) form in blood and urine but had little effect on the passage of the (+) isomer.

I. INTRODUCTION

The biological activity of certain inorganic coordination complexes has been the subject of previous communications (Dwyer *et al.* 1952; Koch *et al.* 1956). The complex cations investigated were coordinately saturated and of high chemical stability and did not contain any specific active groups or centres. Experiments on enzyme systems (Dwyer *et al.* 1952; Koch *et al.* 1956), rat diaphragm and frog sartorius preparations (Shulman, unpublished data), and estimation of their toxicity to mice after intraperitoneal administration (Koch *et al.* 1956) showed that they are potent inhibitors of acetylcholinesterase and possess curare-like activity. The anticholinesterase activity and the toxic action of series of metal complexes of phenanthroline, bipyridine, and terpyridine have been investigated in detail.

It was concluded that the enzyme-inhibitory activity depended on the distribution of the cationic charge of the complex, and, in the asymmetric compounds, on their enantiomeric form. In one group of complexes toxicity paralleled enzyme-inhibitory activity but in other groups of asymmetric complexes there was no such close agreement. Although this result could indicate a different mechanism of action *in vivo* and *in vitro* the possibility that the relative ability of isomers to penetrate animal tissue governed their toxicity could not be excluded. Some

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discrepancies between results obtained *in vivo* and *in vitro* could be explained if the (+) forms penetrated tissues at a greater rate than the (−) forms.

The present work was carried out to investigate this possibility and to obtain general information on the transport and excretion of a representative complex ion in the intact animal.

II. METHODS

(a) *The Preparation of (+) and (−) Tris-1,10-phenanthroline $^{106}\text{Ruthenium}$ (II) Perchlorate [$^{106}\text{Ru}(\text{phen})_3(\text{ClO}_4)_2$]*

Five mc of ^{106}Ru in nitric acid solution, without carrier, was transferred to a small evaporating dish and allowed to stand over solid caustic soda in a desiccator at 20 mm pressure until all the acid had evaporated. The dry material, probably in the form of ruthenyl (IV) nitrate was allowed to undergo exchange for 3 days at room temperature with 2 g of potassium pentachlorohydroxyruthenate (IV) suspended and partly dissolved in 25 ml of 0.1N hydrochloric acid. The mixture was transferred to a 250-ml flask and diluted to 100 ml with distilled water. Phenanthroline (3.5 g) and 5 ml of 10 per cent. sodium hypophosphite were added and the mixture heated in a water-bath at 100°C for 4 hr with occasional shaking. After filtration and addition of solid potassium iodide, the orange-red precipitate of tris-1,10-phenanthroline ruthenium (II) iodide was recrystallized from hot water. The resolution was carried out through the antimonyl tartrate as described previously (Dwyer and Gyarfás 1949). The optical antipodes were isolated as the perchlorates which were recrystallized from hot water.

At 450 μ , equal concentrations of the (+) and (−) forms had the same transmission within 1 per cent. and were assumed to be chemically pure. The ($\pm$) form of the cation was obtained by mixing equal amounts of the (+) and (−) forms.

(b) *Chromatography*

Chromatograms, prepared on 1-cm strips of Whatman No. 1 filter paper, were used to examine the nature of complexes excreted in urine. The ruthenium complexes were identified by their yellow colour on the chromatogram. When the position of ^{106}Ru was to be examined, the chromatograms were covered on both sides with a strip of cellulose tape. The chromatogram, thus protected to avoid contamination, was cut into 5-mm sections and the radioactivity assessed by means of a thin-window Geiger tube and counter.

Preliminary tests using c. 2 μ l of saturated solution of $\text{Ru}(\text{phen})_3(\text{ClO}_4)_2$ in water showed that ethyl alcohol–water–concentrated hydrochloric acid (50 : 48 : 2 v/v) was a suitable solvent for the chromatogram. The (+) and (−) forms of the compound moved with identical R_F values. The addition of 30 μ l of mouse urine to the $\text{Ru}(\text{phen})_3^{++}$ initial spot did not materially affect the R_F value (0.90) of the complex, whether determined by the radioactivity or by the position of the coloured spot. It was considered, therefore, that the salt in mouse urine would not interfere appreciably with the movement of the complexes and undiluted urine was used in the examination of excreted $^{106}\text{Ru}(\text{phen})_3^{++}$ by paper chromatography.

(c) *Measurement of $^{106}\text{Ru}(\text{phen})_3^{++}$*

In preliminary tests different standard amounts of $^{106}\text{Ru}(\text{phen})_3^{++}$ were mixed with varying volumes of blood or urine. These samples were dried at room temperature on planchettes containing a drop of sodium citrate and dilute detergent on which they spread evenly. It was found that the counting rates of the standards were not affected when the volumes of the samples were kept between 0.015 and 0.06 ml. It was concluded, therefore, that absorption in the sample was not critical under these conditions and all experimental material was prepared accordingly for counting. A similar procedure was used to find the conditions at which absorption in the sample was negligible when radioactivity measurements were done on ashed tissue samples. By keeping within these limits the counts obtained from experimental materials could be related to the standards and expressed when necessary in terms of actual amounts of $\text{Ru}(\text{phen})_3^{++}$ in the sample, provided the specific activity of the complex did not change (see Section III(a)).

The use of liquid counters proved unsatisfactory due to the difficulty of removing $^{106}\text{Ru}(\text{phen})_3^{++}$ from the glass even by washing with unlabelled $\text{Ru}(\text{phen})_3^{++}$.

Sufficient counts were taken to give a standard deviation of 6 per cent. of the mean at the lowest counting rates. The accuracy was always greater than this at higher counting rates.

(d) *Collection and Treatment of Biological Materials*

Mice of c. 30 g weight were dosed by intraperitoneal injection. When urine was to be collected the mice were placed in small cages of spirally wound wire so arranged that the urine fell directly on to silicone-coated watch-glasses. At the end of the experiment the mice were anaesthetized with ether, care being taken to collect any urine that was passed, and the bladder opened for the removal of the remaining urine. Urine volumes were made up to 1 ml from which samples for assessing radioactivity were taken.

In early experiments blood was taken from the tail; in later experiments blood was obtained by severing the vessels in the necks of mice anaesthetized with ether.

For assessing the distribution of the complex in the animal tissues after intraperitoneal injection, male albino rats were injected at the rate of $15\text{ }\mu\text{g/g}$ body weight and decapitated after 20 min under ether anaesthesia. The tissues were washed with saline and approximately 0.5-g samples were weighed and placed into nickel crucibles. The samples were dried to a constant weight in a 60°C oven and ground up with sodium carbonate. Repeated small quantities of sodium peroxide were added and the tissues fused by careful heating until white. After the addition of ethylenediaminetetra-acetic acid and citric acid solutions (to avoid precipitation of insoluble nickel and calcium salts) the samples were left to simmer for a short time. On completion of this procedure the ashed tissue was completely water soluble when the pH was adjusted to 7.0. Appropriately diluted samples of these solutions were taken for the determination of radioactivity. All samples were counted at no less than two different dilutions.

III. PROCEDURE AND RESULTS

(a) *The Metabolism of $^{106}\text{Ru}(\text{phen})_3^{++}$*

It was necessary to establish that the complexes were stable biologically, and in particular to show that the radioactive tracer could be used as an indicator for the whole complex. For this purpose mice were injected intraperitoneally with 0.4 ml of (+) or (−) form of the labelled complex at the concentration 0.25 mg/ml and 5×10^5 counts/min/ml.

In different experiments urine was collected for 30 and 150 min. Samples of 3 μl of undiluted urine were placed on chromatograms a little at a time so that the applied spot never exceeded 2 mm in diameter.

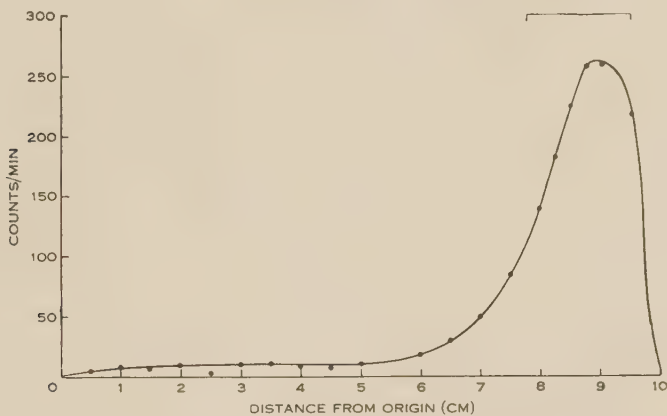


Fig. 1.—Radiochromatogram of urine containing $^{106}\text{Ru}(\text{phen})_3^{++}$. The bracket above the peak indicates the vertical extent and distance from the origin of the yellow spot found on the chromatogram.

The R_F values obtained from such chromatograms were approximately the same, whether obtained by assessing the distribution of the radioactivity or from the distribution of the yellow colour on the whole chromatogram (Fig. 1).

In six experiments the urine and faeces were collected for 24 hr. After this period the mice were killed, and their excised intestines carefully washed out. The combined counts of urine, faeces, and intestinal contents represented 97–99 per cent. of the original dose and 78–91 per cent. was present in the urine. Chromatograms run from these 24-hr urine samples showed a single spot with an R_F value of 0.9.

These experiments allow the following conclusions: At a stage when up to 99 per cent. of the administered radioactivity was recovered the chromatograms still showed only one single spot with the R_F value of the original complex. It seems probable, therefore, that $\text{Ru}(\text{phen})_3^{++}$ was not metabolized, but was excreted unchanged and that ^{106}Ru was a satisfactory label for the compound. Considering this together with the fact that $\text{Ru}(\text{phen})_3(\text{ClO}_4)_2$ does not occur naturally it must be concluded that the specific activity of the complex had not changed during the

experiment. Consequently radioactivity counts, obtained by following the procedure outlined in Section III(c), could be related directly to the standard samples of $^{106}\text{Ru}(\text{phen})_3^{++}$.

(b) *The Relation between Dose Rate and $^{106}\text{Ru}(\text{phen})_3^{++}$ in Blood and Urine*

Mice were injected with appropriate amounts of saline solution containing 250 $\mu\text{g}/\text{ml}$ of the (+), the (−), or the ($\pm$) form of the labelled complex with an activity of 300,000 counts/min/ml. The mice were killed 1 hr after intraperitoneal

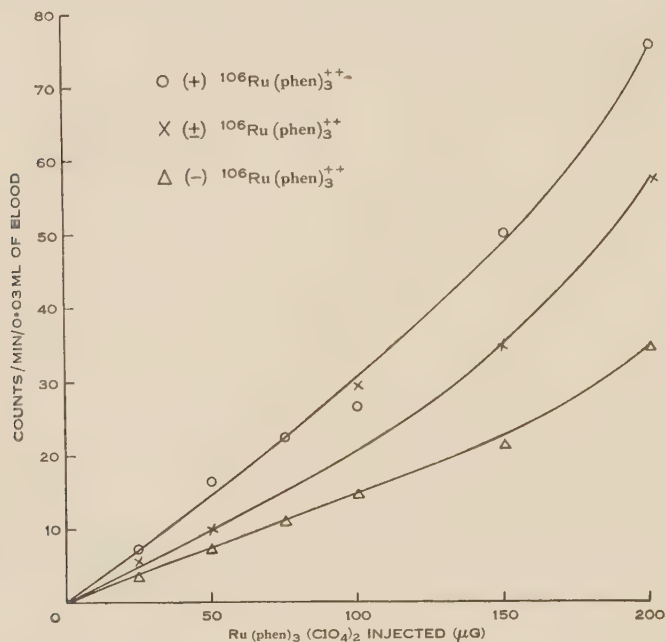


Fig. 2.—Relationship between dose rate and blood levels of $^{106}\text{Ru}(\text{phen})_3^{+++}$ 1 hr after intraperitoneal injection.

injection, and the ^{106}Ru in urine and blood assessed. Results shown in Figure 2 indicate that the (−) form reached the blood in smaller amounts than either the (+) or ($\pm$) forms and that this relationship was consistent in doses from 25 to 200 μg . Higher dose rates could not be examined as the toxic range of the (+) form would have been reached. Similar results were obtained when the urine was examined.

(c) *The Relation between Rate of Excretion and the Concentration in Blood*

Mice were injected intraperitoneally with 0.2 ml of labelled $^{106}\text{Ru}(\text{phen})_3^{+++}$ containing 50 μg of the complex with an activity of 6×10^4 counts/min. Mice were killed at intervals and the radioactivity of samples of blood and urine was measured.

The results obtained with (+) $^{106}\text{Ru}(\text{phen})_3^{+++}$ are shown in Figure 3. With (−) $^{106}\text{Ru}(\text{phen})_3^{+++}$ the concentration found in the blood was about half that obtained

with the (+) form and fell to about $3 \times 10^{-3} \mu\text{g}/0.03 \text{ ml}$ after 90 min. In the same time the output in the urine was about half that of the (+) form. The ($\pm$) mixture gave results intermediate between those of the (+) and (−) forms. Statistical analysis showed that the amounts of the (−) form reaching the blood and urine were significantly lower than that of the (+) or ($\pm$) forms whereas the latter did not differ significantly between themselves.

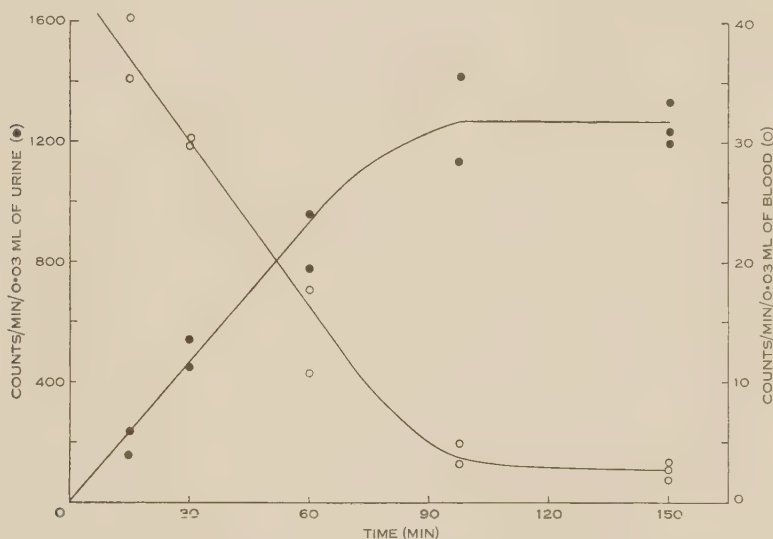


Fig. 3.—Rate of appearance of (+) $^{106}\text{Ru}(\text{phen})_3^{++}$ in blood and urine after intraperitoneal administration of $50 \mu\text{g}$ of the complex salt.

(d) *The Effect of Atropine on $^{106}\text{Ru}(\text{phen})_3^{++}$ Levels in Blood and Urine*

It has been found in earlier experiments (Dwyer *et al.* 1952) that the injection of atropine prior to the complexes potentiated the toxic action of both isomers. It was postulated that atropine increases the permeability of certain tissues to small cations (Kirschner 1953). Therefore it was decided to investigate whether atropine had any effect on the absorption of the complexes.

Atropine sulphate (30 mg/kg) was injected intraperitoneally to mice 15 min before the injection of (+) or (−) $^{106}\text{Ru}(\text{phen})_3^{++}$. The complexes were given in doses of 0.2 ml containing $50 \mu\text{g}$, with a count of $6 \times 10^4/\text{min}$, to 30-g mice by intraperitoneal injection. At intervals the mice were killed, and blood and urine samples were taken for the assay of radioactivity and were compared with samples from control animals which had not been treated with atropine.

As shown in Figure 4, atropine has a pronounced effect on the rate of appearance of (−) $^{106}\text{Ru}(\text{phen})_3^{++}$ in the blood and urine. The results obtained with the (+) form indicated that atropine had little effect on the passage of this isomer into blood and urine.

(e) *The Distribution of $^{106}\text{Ru}(\text{phen})_3^{++}$ between Blood Plasma and Erythrocytes*

One hr after injection of 0.8 ml (200 μg) of the (+) or (−) forms, blood was collected from the vessels in the neck of mice. The red cells were collected by centrifuging and washed three times with ice-cold saline. No radioactivity could be detected in red cells treated in this manner, while counts obtained at the same time from whole blood showed high activity (Fig. 2).

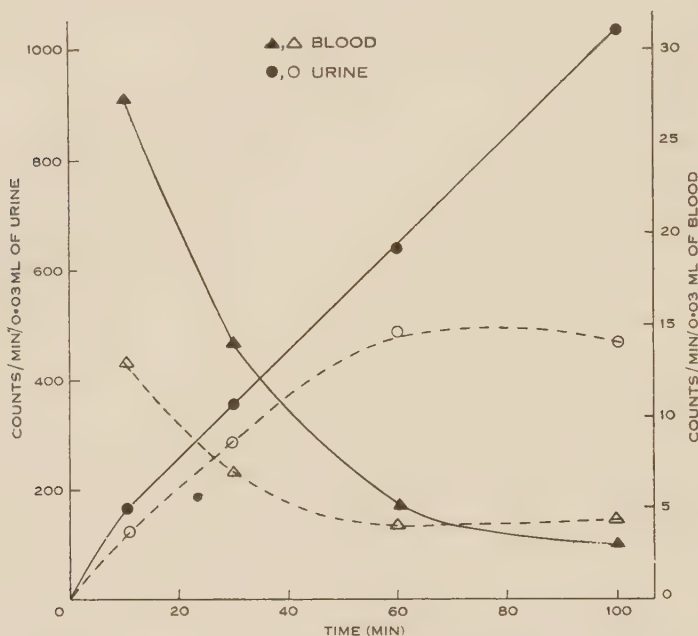


Fig. 4.—Effect of atropine on the rate of appearance of (−) $^{106}\text{Ru}(\text{phen})_3^{++}$ in blood and urine. — Mice which had been pre-treated with atropine. ----- Untreated controls.

(f) *The Distribution of $^{106}\text{Ru}(\text{phen})_3^{++}$ in the Tissues of the Rat*

A group of six male albino rats of c. 300 g weight was injected intraperitoneally with the lethal dose of 15 $\mu\text{g/g}$ body weight of (+) $\text{Ru}(\text{phen})_3^{++}$ and another group with the same, but non-lethal, dose of the (−) form of the complex. The solutions contained 1 mg/ml of the complex in saline, and were adjusted with unlabelled complex in such a way as to give 1.5×10^6 counts/min for 4.5 mg. The rats receiving the (+) form died in approximately 15 min and those receiving (−) form were killed after the same time interval.

When the abdominal cavity was opened unabsorbed quantities of the original injection could be still distinguished by the orange-red colour of the fluid. The tissue samples were collected and dry-ashed as described. Samples of liver, kidney, lung, spleen, pancreas, intestines, suprarenals, testis, heart, diaphragm, skeletal muscles, skin, eye, central nervous system (C.N.S.), blood, and urine were taken for assessment of radioactivity. No radioactivity could be detected in any part of the C.N.S. Varying

small amounts, well below blood level (500–1200 counts/min/g wet weight of tissue), could be found in all the tissues investigated, except liver and kidney which seemed to accumulate greater amounts of the compounds (see Table 1). In a few experiments values as high as those of the liver were found for both diaphragm and pancreas

TABLE I
DISTRIBUTION OF LABELLED (+) AND (–) $\text{Ru}(\text{phen})_3(\text{ClO}_4)_2$ IN SOME TISSUES OF THE RAT

Experiment No.	Weight of Rat (g)	Isomer Injected	Time Elapsed between Injection and Sampling (min)	Activity of Samples			
				Counts/min/g wet wt.		Counts/min/ml	
				Kidney	Liver	Blood	Urine
1	284	(+)	15½	8,000	10,800	2,600	610,000
2	299	(+)	15	13,600	10,250	2,700	401,000
3	301	(+)	12	10,600	9,600	3,600	370,000
4	301	(+)	13½	9,600	8,950	3,100	396,000
5	265	(+)	13	13,400	11,200	3,400	530,000
6	255	(+)	15	15,800	11,500	3,100	470,000
7	278	(–)	13	4,900	5,800	1,600	193,000
8	305	(–)	13	5,750	5,300	2,100	287,000
9	301	(–)	13	6,100	4,350	1,800	207,000
10	299	(–)	15	6,700	6,100	2,100	300,000
11	276	(–)	15	6,900	5,900	1,800	275,000
12	282	(–)	15	7,900	4,750	1,900	304,000

and somewhat lower counts for the spleen. As these high counts in the latter three tissues could not be found repeatedly and were in contrast with the results of the rest of the experiments, it was assumed that they were caused by direct contamination with the injected solution.

IV. DISCUSSION

The chromatographic experiments described above allow an important conclusion: The $\text{Ru}(\text{phen})_3^{++}$ cation is not metabolized by mammalian tissues. The ruthenium is not released from the grip of its chelating molecules. Consequently any observed biological activity cannot be caused by the metal atom or by the chelating agent or any fraction of it, but must be due to the complex cation as a whole. Because no chemical change can be involved, the action of the complex cation must be physical in its nature, as, for example, by adsorption on to negatively charged sites of proteins etc. Obviously in such a process the configuration of the compound could be important, and differences in the behaviour of isomers might be expected.

This would apply also to the penetration of the compounds through cell barriers, and, in fact, the above experiments show that the rate of absorption of the (+) form into the blood stream from the peritoneal cavity is approximately twice that of the (–) form.

Beccari (1941), working on the pharmacological properties, and distribution in tissues, of tris-2, 2'-bipyridine iron (II) sulphate, which is a complex having the same biological activity and similar chemical properties to $\text{Ru(phen)}_3(\text{ClO}_4)_2$ (Koch *et al.* 1956), found that the toxicity depended only on its concentration in the blood stream. Our studies show that Ru(phen)_3^{++} was excreted rapidly, and did not appear to be accumulated considerably by any tissue other than kidney and liver, neither of which was involved in the immediate curare-like toxic action of the complex.

As the concentration of the complex in the blood stream depends primarily on its rate of absorption, and as our results showed that the (+) isomer was more rapidly absorbed than the (−) isomer, the higher toxicity of the former is to be expected.

Our findings on the distribution of $^{106}\text{Ru(phen)}_3^{++}$ in the tissues are in agreement with Beccari's results with the iron complex.

V. ACKNOWLEDGMENTS

The authors would like to express their gratitude to Mr. J. W. Hogarth for the method of dry ashing; to the Division of Mathematical Statistics, C.S.I.R.O., for the evaluation of the experimental data; and to Miss Judith Smith for much technical assistance. Special acknowledgment is due to Mr. R. A. Simmonds who played a major part in the experimental work.

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PROTHROMBOPLASTIN ACTIVITIES OF SOME MAMMALIAN PLASMAS AND SERA

By P. FANTL* and A. G. MARR*

[*Manuscript received March 27, 1957*]

Summary

The rate of formation of thromboplastin was determined in several mammalian plasmas and sera.

A marked difference in stability of blood thromboplastin was observed. Catechol had a stabilizing effect on blood thromboplastin.

α - and β -prothromboplastin (anti-haemophilic factor and PTC or Christmas factor) showed no species specificity when combined with heterologous clotting components.

The plasma of man, rabbit, guinea pig, and rat showed a similar range of α - and β -prothromboplastin activities. Dog plasma showed a higher activity.

In addition to α - and β -prothromboplastin other factors are required for efficient thromboplastin formation; one or more of these are present in serum and these show species specificity.

I. INTRODUCTION

Recently we described (Fantl, Sawers, and Marr 1956) a haemorrhagic tendency of a male bleeder whose plasma contained an anticoagulant which inhibited specifically β -prothromboplastin (PTC or Christmas factor) activity of normal human plasma. The anticoagulant was very likely an antibody produced by sensitization of the patient by blood transfusions. The lack of anticoagulant activity on animal plasma could be explained by the specificity of the antibody or could be due to a considerably higher concentration of β -prothromboplastin in animal than human plasma. In the latter case the anticoagulant would neutralize a fraction of β -prothromboplastin and the remaining excess would mask the anticoagulant activity. Therefore, activities and the species specificity of prothromboplastins of human and animal plasmas were determined.

II. METHODS AND MATERIALS

Human blood was obtained by venipuncture from normal donors. Sheep blood was obtained from the jugular vein. Other animals were anaesthetized with ether. Rabbit blood was obtained from the exposed carotid artery through a siliconized cannula. Guinea pig and rat blood were taken from one of the ventricles. Dog blood was obtained from the femoral artery. As anticoagulants 0.1M sodium oxalate or 0.13M trisodium citrate were used. One volume of anticoagulant was mixed with nine volumes of blood. In the case of guinea pig and rat blood anticoagulant was already in the syringe. The thromboplastin generation test was carried

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out according to Biggs and Douglas (1953), the modification of Bell and Alton being adopted (1954), except that oxalated plasma treated with barium sulphate (Ba-plasma) was used instead of aluminium hydroxide-treated citrated plasma. Phospholipid was prepared from sheep brain according to Eagle (1935). The partial thromboplastin test was carried out according to Langdell, Wagner, and Brinkhous (1953).

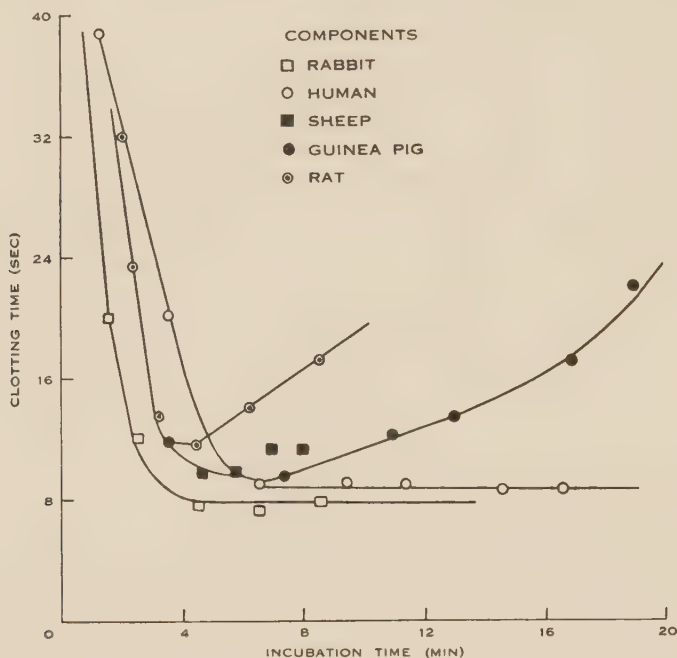


Fig. 1.—Thromboplastin formation in homologous plasma and serum. The reaction mixtures contained 0.12 ml Ba-plasma, 0.06 ml homologous serum, 0.6 ml phospholipid suspension, 0.6 ml 0.025M CaCl_2 , 1.02 ml 0.15M NaCl; substrate: homologous plasma. Technique of Biggs and Douglas (1953).

α -Prothromboplastin deficient blood (α -haemophilia) and β -prothromboplastin deficient blood (PTC, Christmas disease) were taken from the respective patients and collected in a solution containing 0.104M trisodium citrate and 0.053M citric acid. The plasmas were stored at -15°C . SPCA test was carried out according to de Vries, Alexander, and Goldstein (1949). Veronal buffer of ionic strength 0.15 was prepared according to Michaelis (1930).

III. RESULTS

Thromboplastin formation was measured in reaction mixtures which consisted of homologous plasma and serum components. The results are given in Figure 1 where it can be seen that if homologous blood components with the exception of phospholipids are used maximum thromboplastin activity occurred after 5–6 min

incubation time. The shortest clotting time was between 7 and 11 sec. A marked difference in stability of thromboplastin was apparent; whereas the thromboplastins of rabbit and that of man were stable for some minutes, guinea pig and rat thromboplastin activity diminished after 1 min.

It should be pointed out that whereas the blood specimens from man, rabbit, and sheep were obtained with the least trauma, the guinea pig and rat blood were certainly contaminated with tissue thromboplastin.

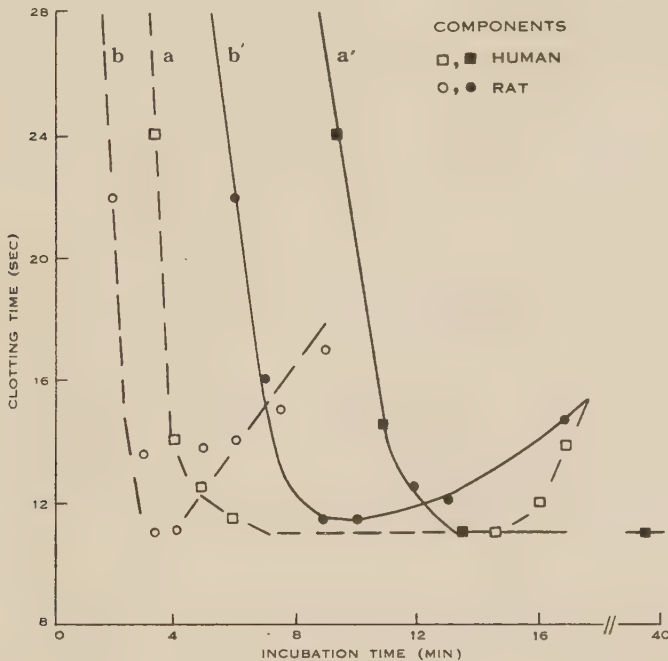


Fig. 2.—Influence of catechol on thromboplastin formation and stability. In *a* and *b* aqueous 0.025M CaCl_2 was used. In *a'* and *b'* 0.025M CaCl_2 in veronal buffer, pH 7.2, which contained 0.18M catechol, was used. Otherwise reaction mixtures and technique similar to that in Figure 1.

Spaet and Garner (1955) have indicated a certain similarity between the thromboplastin inactivator present in plasma and antithrombin. Since it has been found that phenols inactivate antithrombin (Fantl 1954), the influence of catechol on blood thromboplastin was determined. The results are given in Figure 2.

It is apparent from Figure 2 that although there is a lag in the formation of thromboplastin in the presence of catechol, this substance has some stabilizing effect on thromboplastin which is far more pronounced with human material than with that of the rat. Maximum thromboplastin formation and stability of human thromboplastin was determined in a number of instances and the results are given in Table 1.

The results in Table 1 indicate that veronal buffers at pH 6·8–7·5 did not influence the result although a lag period in the formation of thromboplastin is

TABLE 1
THROMBOPLASTIN FORMATION AND STABILITY*

The values represent average results, variations are given in brackets

Thromboplastin Formation in the Presence of:	Incubation Time for Max. Activity (min)	Shortest Clotting Time (sec)	Period of Constant Activity (min)
0·025M aqueous CaCl_2	7 (6·5–8)	11 (10–12)	14 (6–20)
0·025M aqueous CaCl_2 in veronal buffers, pH 6·8–7·5	12 (11–15)	11 (10–13)	12 (9–17)
0·025M aqueous CaCl_2 in veronal buffer, pH 7·2, containing 0·18M catechol	27 (17–28)	10–11	30 (20–31)

*Technique of Bell and Alton (1954), temperature 28°C.

apparent. This is very pronounced in the presence of catechol, but the stabilizing effect of catechol is quite noticeable.

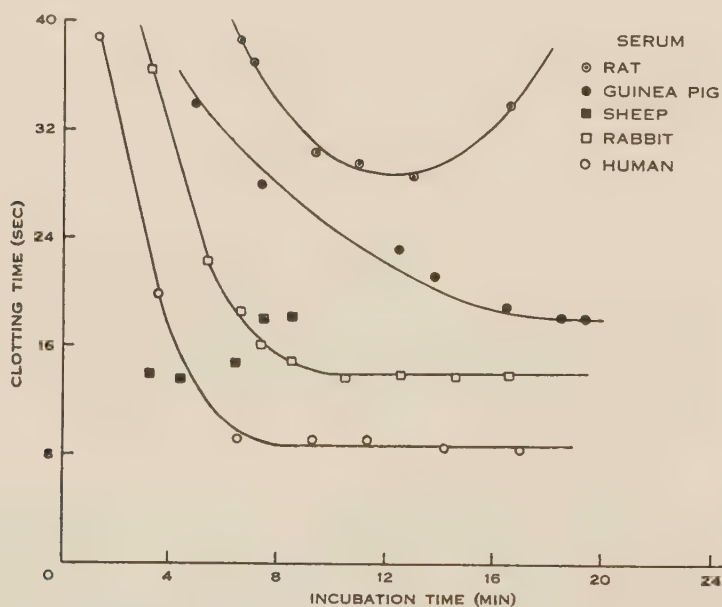


Fig. 3.—Thromboplastin formation with human Ba-plasma and various mammalian sera. Reaction mixtures and technique similar to that in Figure 1.

In the following experiments the ability to form thromboplastin was tested in reaction mixtures containing human Ba-plasma and human or animal sera. Results are given in Figure 3, where it can be seen that thromboplastins formed

from human Ba-plasma, and heterologous serum were less active than thromboplastins formed from homologous human blood components. In other experiments different brain phospholipids and also human platelets were used as a source of phospholipids; substantially similar results to those in Figure 3 were obtained.

TABLE 2
THROMBOPLASTINS TESTED ON HOMOLOGOUS AND HETEROLOGOUS PLASMA*

Source of Thromboplastin	Shortest Clotting Time (sec) Obtained with Substrate Plasma of:				
	Man	Rabbit	Sheep	Guinea Pig	Rat
Man	9	8	—	10	9
Rabbit	8	7	—	—	—
Sheep	11	—	11	—	—
Guinea pig	12	—	—	10	11
Rat	11	—	—	12	11

*Reaction mixtures and technique similar to that of Figure 1.

From the activities of a series of dilutions of human thromboplastin it was estimated that the percentage relative activity of thromboplastins formed from human Ba-plasma in the presence of serum was: human 100, rabbit and sheep 30–45, guinea pig 20–30, rat 5–15. These values are considerably lower than those found in homologous reaction mixtures (Fig. 1). The low potency of the thromboplastins

TABLE 3
 α -PROTHROMBOPLASTIN ACTIVITY OF MAMMALIAN PLASMA
Each animal plasma was compared with a different normal human plasma

Normal Plasma Source	Thromboplastin Generation Test*	Partial Thromboplastin Test†
Man	100	100
Rabbit	200	—
Guinea pig	150	250
Rat	—	100–200
Dog	—	1400

*The same technique as in Figure 1.

†Technique of Langdell *et al.* (1953).

formed in the presence of heterologous sera could be due to species specificity of the thromboplastins or to a reduced activity of any one of the serum factors, or of the Ba-plasma towards the heterologous components required for thromboplastin formation.

In order to see whether the thromboplastins showed species specificity, the various mammalian plasma thromboplastins were produced by incubating serum and homologous Ba-plasma; the reaction mixtures were tested on homologous as well as heterologous plasma. Results are given in Table 2. From this table it is seen that although there were differences in thromboplastin activity among the various thromboplastins, the reaction mixtures showed the same clotting times with homologous as with heterologous plasma as substrate. Apparently there was no species specificity of blood thromboplastin.

Independent determinations of each factor required for the formation of blood thromboplastin were carried out in order to find which factor showed species specificity.

TABLE 4
 β -PROTHROMBOPLASTIN ACTIVITY OF MAMMALIAN PLASMA AND SERA*

Source of Normal Plasma or Serum	Thromboplastin Generation Test	Partial Thromboplastin Test
Man	100	100
Rabbit	50-100	250
Guinea pig	50-100	—
Rat	100	100
Dog	—	400

*The same techniques as those in Table 3.

The α -prothromboplastin activity was estimated from the results of two independent techniques in which the correcting influence of normal animal or human plasma added to the plasma of patients with a complete deficiency of α -prothromboplastin was measured. The results are given in Table 3, and indicate that the plasmas of man, rabbit, guinea pig, and rat were not significantly different in their α -prothromboplastin activity. Dog plasma showed higher activity.

β -Prothromboplastin activity of the various plasma and sera was tested for ability to restore the deficiency of plasma or sera of patients with a complete lack of β -prothromboplastin. These results are given in Table 4. They show that the plasma or sera of normal man, rabbit, guinea pig, and rat are not significantly different in their β -prothromboplastin activity. Dog plasma had a higher activity. It will be noted that the guinea pig or rat serum was considerably more active in the tests given in Table 4 than in the experiments in Figure 3.

There was no inhibition of thromboplastin formation in mixtures of guinea pig or rat serum with human serum and human Ba-plasma. Therefore the low yield of thromboplastin from human Ba-plasma and animal sera cannot be due to inhibitors of thromboplastin formation in the animal sera.

It would seem therefore that neither α - nor β -prothromboplastin shows a decreased affinity to the other factors in reaction mixtures containing heterologous

clotting components. It appears that the species specificity noted in Figure 3 was due to a different factor present in serum which may belong to the proconvertin-convertin complex.

As patients with such deficiencies (SPCA, proconvertin, factor 7, Stewart factor) were not available, the appropriate tests could not be carried out. Instead the animal sera were tested for serum accelerator factors in combination with brain thromboplastin with the technique of de Vries *et al.* (1949). The results are

TABLE 5
ACCELERATOR ACTIVITY OF MAMMALIAN SERA ON THE CLOTTING OF HOMOLOGOUS AND HETEROLOGOUS PLASMA*

In each case 0.01 ml of 0.15M NaCl or serum was added at the concentration indicated; to the mixtures 0.1 ml 0.025M CaCl_2 was added

Addition	Clotting Time (sec) Obtained with:					
	Human Components† plus Addition at Levels:		Rabbit Components‡ plus Addition at Levels:		Guinea Pig Components§ plus Addition at Levels:	
	10%	5%	10%	5%	10%	5%
0.15M NaCl	60	60	32	32	37	37
Human serum	23	26	25	28	43	40
Rabbit serum	30	37	23	27	38	—
Sheep serum	26	30	28	31	36	—
Guinea pig serum	29	34	32	33	30	32

*Technique of de Vries *et al.* (1949).

† 0.0855 ml human Ba-plasma, 0.0045 ml human oxalated plasma, 0.1 ml 6% human brain suspension in 0.15M NaCl.

‡ 0.08775 ml rabbit Ba-plasma, 0.00225 ml rabbit oxalated plasma, 0.1 ml 3% rabbit brain suspension in 0.15M NaCl.

§ 0.08775 ml guinea pig Ba-plasma, 0.00225 ml guinea pig oxalated plasma, 0.1 ml 3% guinea pig brain suspension in 0.15M NaCl.

listed in Table 5, and indicate that the shortest clotting times were observed in reaction mixtures which contained homologous components only. These results in conjunction with the previous observations indicate that it is a serum factor of the proconvertin-convertin complex which shows species specificity.

IV. DISCUSSION

A comparison of the rate of formation and yield of thromboplastin in homologous and heterologous clotting systems indicated that only some of the clotting factors are species specific. This seems remarkable because most of the clotting factors are proteins and one would expect that all or none of them show greater

affinity to homologous than to heterologous components of the clotting system. Several mammalian plasmas with the exception of that of the dog had a similar activity to normal human plasma in correcting the clotting defect of patients with α - or β -haemophilia.

Other workers found α -prothromboplastin activity of plasma as follows: man 100, rabbit 100, rat 100, dog 200, beef-cattle 400 (Richards and Spaet 1956); beef-cattle 1300 (Biggs, Eveling, and Richards 1955), rabbit 500 (Egli and Kessler 1955). The β -component has apparently not been investigated by other workers. Since it is unlikely that thromboplastin precursors show higher affinity to foreign proteins than to their own plasma components it seems that the observed α - or β -prothromboplastin activity irrespective of source is only dependent upon concentration. α - and β -prothromboplastin are therefore not species specific.

This suggests that the blood thromboplastin precursors contain active centres for the coagulation process which are distinct from those essential for antigenicity, since the inhibitor of human β -prothromboplastin activity which was present in the blood of a male bleeder (Fantl *et al.* 1956) did not inactivate the β -prothromboplastins of the plasma of several animals.

In addition to the two thromboplastin precursors mentioned other factors are required for optimum thromboplastin formation. One of them, prothrombin accelerator (proaccelerin, labile factor, factor 5) shows usually higher activity in animal than in human plasma and it has been noticed elsewhere (Fantl and Ward, unpublished data) that this clotting factor is not species specific. Other components required for optimum thromboplastin formation are present in the proconvertin-convertin complex. This has not been resolved into individual constituents, and it is therefore not possible to indicate which one of them is involved in thromboplastin formation. However the combination of heterologous sera with human Ba-plasma resulted in thromboplastins of lower potency, than expected from the α - and β -prothromboplastin activities. This indicated not only that additional serum factors are required for efficient thromboplastin formation but also that one or more of the unidentified factors show pronounced species specificity.

Thromboplastins of organ extracts usually give shorter clotting times with homologous than with heterologous substrates. This was not apparent in experiments where blood thromboplastin containing reaction mixtures were tested on human and heterologous plasma. The reason for this apparent lack of species specificity is probably due to the presence of serum. This is comparable to the observation made by Burstein (1950) and by Mann and Hurn (1952) who found that incubation of brain extracts with homologous sera abolished species specificity.

V. ACKNOWLEDGMENT

This work was carried out with the support of a grant from the National Health and Medical Research Council, Canberra.

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STUDIES IN ANAPHYLAXIS

I. QUANTITATIVE SCORES FOR ANAPHYLAXIS IN MICE

By P. J. CLARINGBOLD* and W. R. SOBEY†

[*Manuscript received February 22, 1957*]

Summary

Quantitative scores for anaphylactic symptoms in mice have been estimated. The usefulness of these scores is discussed, and their superiority over the commonly used quantal score (alive or dead) is emphasized.

I. INTRODUCTION

The symptoms of anaphylaxis in mice have been described in detail by a number of workers, e.g. Weiser, Golub, and Hamre (1941), McMaster *et al.* (1954), and Cameron (1956). After a shocking dose, symptoms range from no apparent effect to death with many intermediate manifestations. While it is apparent that the symptoms may be qualitatively graded in order of increasing severity, no direct quantitative measure of effect is available. In view of the relative resistance of the mouse to anaphylaxis when compared with the guinea pig, and also in the interest of precision in experiments, it is of importance that quantitative scores be obtained for easily recognizable symptoms. Fisher (1948) has given an approach to the problem which is considered in this paper.

II. EXPERIMENTAL METHODS

(a) *Mice*

Female albino mice weighing 20 g and 12 weeks old, obtained from a randomly bred strain, were used in this work. The mice were housed four per box and given standard mouse cubes and water *ad lib.*

(b) *Antigen*

Bovine plasma albumin (Armour, fraction 5) was used as an antigen. The total sensitizing dose was administered intraperitoneally in two injections, each in 0.5 ml N saline, spaced 1 week apart. The shocking dose was administered 1 week after the second sensitizing dose, and was given intravenously in 0.5 ml N saline.

(c) *Experimental*

The experiment was of the 4² factorial type (Cochran and Cox 1950) with four mice in each treatment group (i.e. 64 mice were used in the experiment). One variable

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studied was the sensitizing dose given at four different levels. The other variable, the shocking dose, was also given at four levels. The experimental design and results obtained are given in Table 1.

TABLE 1
FREQUENCIES OF THE SYMPTOMS IN THE 16 TREATMENT GROUPS
The animals showing no symptoms (θ) are omitted

Shocking Dose (mg)	Sensitizing Dose (mg)				Total
	12.5	25	50	100	
0.05	$2x + y + a$	$2y + 2a$	$y + 3z$	$4a$	$2x + 4y + 3z + 7a$
0.5	$x + 3y$	$y + 2z + a$	$2y + z + a$	$y + z + 2a$	$x + 7y + 4z + 4a$
5	$2x + y$	$x + y + a$	$3y$	$x + 3y$	$4x + 8y + a$
50	y	$x + y$	$x + 3y$	$4y$	$2x + 9y$
Total	$5x + 6y + a$	$2x + 5y + 2z + 4a$	$x + 9y + 4z + a$	$x + 8y + z + 6a$	$9x + 28y + 7z + 12a$

(d) *Symptoms Chosen for Quantitative Evaluation*

Designation	Description
θ	No effect.
x	Animal exhibits vigorous scratching of ears and face and nibbling of anal region.
y	Animal displays absence of any desire to move, huddles in a corner, with laboured breathing and infrequent convulsive movements. The animal will move when stimulated.
z	Paralysis, partial or complete. The animal does not move when stimulated, or moves slowly, dragging its hindquarters.
a	Death.

III. STATISTICAL ANALYSIS

Analysis of variance of the data in Table 1 may be made by treating the observations θ , x , y , z , and a as if they were ordinary numbers. This process is made general by the adoption of the notation of matrices and quadratic forms. Following the analysis of variance, estimates of the indeterminate symbols may be obtained subject to restrictions imposed by, and depending on, the requirements of the experimenter. For example, scores may be obtained which maximize the dependence of response on sensitizing dose, or alternatively scores could be obtained which minimized interaction between shocking and sensitizing dose. As the investigations which follow explore the relationship between dose and response it was decided that a score which maximized the dependence of response on log-linear increment of both sensitizing and shocking doses was appropriate to investigate first.

The following sums of squares and cross products were therefore calculated:

	Matrix				D.F.
	<i>x</i>	<i>y</i>	<i>z</i>	<i>a</i>	
Total sum of squares ($\times 64$)	<i>x</i>	<i>y</i>	<i>z</i>	<i>a</i>	64
	<i>y</i>				
	<i>z</i>				
	<i>a</i>				
Correction factor ($\times 64$)	<i>x</i>	<i>y</i>	<i>z</i>	<i>a</i>	1
	<i>y</i>				
	<i>z</i>				
	<i>a</i>				
Linear regression on sensitizing dose ($\times 320$)	<i>x</i>	<i>y</i>	<i>z</i>	<i>a</i>	1
	<i>y</i>				
	<i>z</i>				
	<i>a</i>				
Linear regression on shocking dose ($\times 320$)	<i>x</i>	<i>y</i>	<i>z</i>	<i>a</i>	1
	<i>y</i>				
	<i>z</i>				
	<i>a</i>				

The multipliers (shown in brackets) were introduced to preserve whole numbers. Correcting the total sum of squares for the mean, adding the two sums of squares we wish to maximize, and reducing the results to a common multiplier gives:

	Matrix				D.F.
	<i>x</i>	<i>y</i>	<i>z</i>	<i>a</i>	
$L =$ (sum of squares for linear regressions ($\times 320$))	<i>x</i>	<i>y</i>	<i>z</i>	<i>a</i>	2
	<i>y</i>				
	<i>z</i>				
	<i>a</i>				
$T =$ (total sum of squares corrected for mean ($\times 320$))	<i>x</i>	<i>y</i>	<i>z</i>	<i>a</i>	63
	<i>y</i>				
	<i>z</i>				
	<i>a</i>				

The calculation of scores requires solution of the matrix equation

$$\mathbf{b}'[\mathbf{L} - \theta \mathbf{T}] = 0,$$

where $\mathbf{b}'$ is a vector of estimates (the score), and θ is a root of the determinantal equation (Rao 1952)

$$|\mathbf{L} - \theta \mathbf{T}| = 0.$$

Numerical solution of the equations may be obtained by the method of divided differences (Whittaker and Robinson 1949), and by various matrix procedures (Rao 1952). Since solution of equations of this type has been programmed for automatic computers the computation was carried out on the SILLIAC.

Since a sum of squares with two degrees of freedom is maximized, only two latent roots will differ from zero. Barlett (1947) has given a test of significance of latent roots obtained in the above equations (see Table 2). Only one latent root is found

TABLE 2
LATENT ROOTS AND THEIR TEST OF SIGNIFICANCE

Latent Root	D.F.	χ^2	<i>P</i>
$\theta_1 = 0.6377$	5	60	<0.001
$\theta_2 = 0.0705$	3	4.3	0.3-0.2

significant here. Therefore all the information in the data is contained in the scores corresponding with this root, namely

$$0 = 0.000, x = 0.286, y = 0.365, z = 0.591, a = 0.654.$$

The scores are calculated subject to the condition of orthogonality and, as a result, the sum of squares is unity, i.e.

$$0^2 + x^2 + y^2 + z^2 + a^2 = 1.$$

These scores may be multiplied or divided by constants for convenience in use. As an example of the use of this scoring method for responses, the data of Table 1 have been analysed using these estimated scores (see Table 3).

TABLE 3
ANALYSIS OF VARIANCE OF THE DATA OF TABLE 1 USING THE ESTIMATED SCORES

Source of Variation	D.F.	Mean Square	<i>F</i>
Shocking dose	(3)		
Linear	1	8529.5	40.8***
Remainder	2	368.4	1.8
Sensitizing dose	(3)		
Linear	1	3668.7	17.5***
Remainder	2	121.9	<1
Interactions	9	130.3	<1
Error	48	209.2	

****P* < 0.001.

IV. DISCUSSION

Mice, generally regarded as being more refractory than guinea pigs to anaphylactic shock, may well prove to be useful for quantitative studies since the symptoms displayed by them can be usefully scored. Arbitrary scores based on some

assumed nature of the underlying causes of variation may be applied but these will result in loss of statistical efficiency unless they approach the estimated values closely. The significance of any difference between arbitrary and estimated scores may be tested (Fisher 1948; Biggers and Claringbold 1954). The greater efficiency of a quantitative score over a quantal (alive or dead) score is well illustrated in the present experiment, which would have proved quite inconclusive had a quantal score been employed.

Examination of the estimated scores shows a disproportionate gap between 0 and x , accounting for approximately 43 per cent. of the scale. Any symptoms which could be identified before the onset of x would help provide a more efficient score. In this Laboratory where relatively large numbers of mice are being used in experiments, the emphasis must be on ease of recognizing symptoms. Those given above exhaust, in our experience, such easily recognizable symptoms.

In common with the quantal score, any score which depends on symptoms of the general nature described above theoretically suffers from many sources of variability, although this, of course, may have little bearing on the practical utility of an estimated score. Thus, for example, there may occur genetic differences in the sensitivity of the nasal mucosa to anaphylaxis. In a sensitive line, the symptom designated x could then score at about 20 per cent. instead of the present 43 per cent. of the scale. Effects of this type must be constantly borne in mind when developing the use of scores, particularly in comparisons of genetically different lines.

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CORRECTION FOR REGRESSION ON A SECONDARY TRAIT AS A METHOD OF INCREASING THE EFFICIENCY OF SELECTIVE BREEDING

By R. OSBORNE*

[Manuscript received March 27, 1957]

Summary

A revised estimate is given for the efficiency of a selection index investigated by Rendel (1954) which corrects the variations in a particular trait for linear regression on another.

I. INTRODUCTION

A recent report (Rendel 1954) suggested that the efficiency of selective breeding for a trait of incomplete heritability may be increased by basing selection on an index which corrects the variations of the primary trait for measurable variations introduced by environmental agencies. It was shown that if trait A is correlated only environmentally with B and B has no economic value, the optimum index for improvement of A as a linear combination of A and B is

$$I = A - bB,$$

where b is the phenotypic regression of A on B . The purpose of this note is to re-examine the efficiency of such an index in the case discussed by Rendel when A and B are also genetically correlated.

II. THEORY

The genetic gain in character A when selection is based on an index I is given by $R_{GAI}\sigma_{GA}$, where R_{GAI} is the correlation between I and genotype of A , σ_{GA} is the genetic standard deviation of A , and the gains are measured per unit standard deviation in the selection differential (Hazel 1943). Here, of course, the usual assumptions are made regarding additivity and independence of genetic and environmental variations. If individual phenotype A is the criterion of selection, then

$$R_{GAI} = R_{GAA} = h_A,$$

where h_A^2 is the heritability of A . Thus the gain from phenotypic selection is $h_A\sigma_{GA}$ and the efficiency of index selection relative to this is

$$R_{GAI}/h_A = \text{cov}(G_A, I)/h_A\sigma_{GA}\sigma_I.$$

Since

$$I = A - bB,$$

then

$$\text{cov}(G_A, I) = \text{cov}(G_A, A - bB) = \text{cov}(G_A, G_A - bG_B) = \sigma_{GA}^2 - br_G\sigma_{GA}\sigma_{GB},$$

which reduces to

$$\sigma_{GA}^2(1 - r_P r_G h_B/h_A),$$

where r_P and r_G are the phenotypic and genetic correlations of A and B and h_B^2 is

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the heritability of B . The well-known result of correcting the variance of one variable for regression on another gives $\sigma_I = \sigma_A \sqrt{1 - r_P^2}$. Since also $h_A = \sigma_{G_A} / \sigma_A$ the relative efficiency of index selection reduces to

$$E = \frac{1 - r_P r_G h_B / h_A}{\sqrt{1 - r_P^2}},$$

rather than $\frac{1 - r_G^2}{\sqrt{1 - r_P^2}}$ as given by Rendel.

It may be noted that Rendel's result would follow if b in the expression $\text{cov}(G_A, G_A - b_{G_B})$ were the genetic regression coefficient. There are, however, other considerations involved with respect to his analysis. The gain from phenotypic selection for A may be written as $h_A^2 \sigma_A$. It was then assumed, or at least implied, that the gain in A from selection on the index is $h_I^2 \sigma_I$, the product of heritability and standard deviation of the index. Heritability of the index is the ratio of its genetic to phenotypic variance, the latter being given, as above, by $\sigma_I^2 = \sigma_A^2 (1 - r_P^2)$. It does not follow that genetic variance of the index is $\sigma_{G_I}^2 = \sigma_{G_A}^2 (1 - r_G^2)$, since once again the genetic regression is not used in the correction $I = A - bB$. The true value is

$$\begin{aligned} \sigma_{G_I}^2 &= \sigma_{G_A}^2 + b^2 \sigma_{G_B}^2 - 2b \text{cov } G_A G_B \\ &= \sigma_{G_A}^2 (1 + r_P^2 h_B^2 / h_A^2 - 2r_P r_G h_B / h_A). \end{aligned}$$

From these expressions the true value of h_I^2 may be derived. However, it must be emphasized that $h_I^2 \sigma_I$ measures the gain in the index and not in A . Gain in A is given by $b_{G_A G_I} h_I^2 \sigma_I$, where $b_{G_A G_I}$ is the regression of genotype of A on genotype of I . But

$$b_{G_A G_I} h_I^2 \sigma_I = \frac{\text{cov}(G_A, G_I)}{\sigma_{G_I}^2} \cdot \frac{\sigma_{G_I}^2}{\sigma_I} = \frac{\text{cov}(G_A, G_I)}{\sigma_I},$$

so that estimation of $\sigma_{G_I}^2$ is unnecessary. Since $\text{cov}(G_A, G_I) = \text{cov}(G_A, I)$ the gain from index selection is $R_{G_A I} \sigma_G$ as before.

The corrected formula may give either a greater or lesser efficiency than that indicated by Rendel, but it is evident that judicious consideration of phenotypic and genetic correlations and heritabilities of the traits concerned is necessary to evaluate the precision of the method—not only whether it exceeds that of phenotypic selection but whether the relative gain is worth while compared with that from optimum indices for genetically correlated traits.

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INHERITANCE OF ANTIBODY RESPONSE

III. HERITABILITY OF RESPONSE TO SHEEP RED CELLS

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[Manuscript received January 15, 1957]

Summary

The heritability of antibody response to a priming dose of sheep red cells is 41.8 ± 19.4 per cent. and to a secondary dose -7.4 ± 20.7 per cent.

With this antigen it would appear that the factors governing response to a priming dose are considerably different from those governing response to a secondary dose.

It is stressed that adoption of a block replicated experimental design with this experimental material results in considerable gains in precision of experimentation.

I. INTRODUCTION

In the first paper of this series (Sobey and Adams 1955) an estimate of heritability (h^2) of response to sheep red cells (S.R.C.) was obtained using the well-known technique of mid-parent offspring regression. In obtaining this estimate 228 parents and 456 offspring were tested. At that time it was not known that considerable secular variation in response occurred, and as a result the estimate suffered a large sampling error. One purpose of this paper is to study a different approach to the estimation of h^2 using an experimental design which enables the elimination of the secular component, and also the study of other causes of variation.

II. MATERIALS AND METHODS

(a) Serological Technique

Methods of preparing red cells for injection, collection of immune serum and complement, and the routine of testing immune serum have been described in a previous paper (Sobey and Adams 1955).

(b) Preparation of Animals

In all, 256 female albino mice were used in this investigation; of these, 64 were dams obtained from a line of mice selected for increased sensitivity to oestrogens for six generations (Biggers and Claringbold 1955), and another 64 were dams obtained from a line selected for decreased sensitivity. The remaining 128 mice were daughters of these dams (one daughter per dam) by sires from the two selection lines. The basic unit in the experimental design is a daughter-dam pair of animals.

The mice were housed in 64 boxes, four mice per box, and were supplied standard mouse cubes and water *ad lib.* The dams of each line occupied 16 boxes with

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four dams selected at random in each box. The boxes were serially numbered. Individual dams were identified both by an ear clip and a colour code. Thus each dam is identified by her box number and her ear clip or colour. The daughter corresponding to a particular dam was put into a box similarly numbered, and received the same ear clip and colour coding as her dam. To distinguish boxes of dams from those of daughters, the box card colour was varied and dams and daughters were clipped in opposite ears. Subsequent manipulations were carried out with daughter-dam box pairs as units, boxes of daughters being treated in parallel with boxes of dams.

(c) *Experimental Design*

Since it was impossible to determine the antibody responses of the 256 animals simultaneously the experiment was carried out in four blocks each of 32 dams and their corresponding daughters, i.e. of 32 experimental units. The treatment of the first block will be described in detail. The remaining blocks are simply replicates of this, the treatment of successive blocks being started at weekly intervals.

TABLE 1
SCHEMATIC LAYOUT OF THE EXPERIMENTAL TREATMENT OF ONE BLOCK

	High-selection Line				Low-selection Line			
	Intravenous Route		Intraperitoneal Route		Intravenous Route		Intraperitoneal Route	
Dose (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Dose (%)	0.1	0.01	0.1	0.01	0.1	0.01	0.1	0.01
No. of units	4	4	4	4	4	4	4	4

Each block of 32 experimental units was made up of 16 units from each oestrogen-selection line. Of each 16 units, eight were injected with S.R.C. by the intravenous and eight by the intraperitoneal route. Two levels of dose were given to each subgroup of eight units, four units receiving 0.5 ml of a 0.1 per cent. suspension of S.R.C. and four a 0.01 per cent. suspension. The layout of the experimental treatment of one block is shown in Table 1.

The antibody responses of the animals were measured 6 days after the injection of the antigen using the standard technique.

The experiment for the study of primary response is thus a 4×2^3 factorial experiment with four experimental units per treatment combination. Two dependent variables were measured in each experimental unit, namely the responses of the dams and of the daughters. The body weight of all animals was measured to the nearest half gram at the time of testing as an additional concomitant variable. Thus a correlation between response of daughter and dam could be calculated independent of the effect on response of line, dose, route of injection, weight, and time of testing.

Four weeks after receiving the primary dose of antigen, a second injection of S.R.C. was given. The four experimental units within each treatment combination described above and shown schematically in Figure 1 were allocated to an additional four treatment combinations. Two of the units were injected by the intraperitoneal route and two by the intravenous route, a high dose and a low dose being given to one unit by each route. Six days later the secondary response of the animals was determined.

The experimental design for secondary responses is thus a 4×2^5 factorial experiment with one experimental unit per treatment combination. The factors are blocks (*Bl*), lines (*L*), primary injection route (*Rp*), primary dose (*Dp*), secondary injection route (*Rs*), and secondary dose (*Ds*). Since all animals were mature at

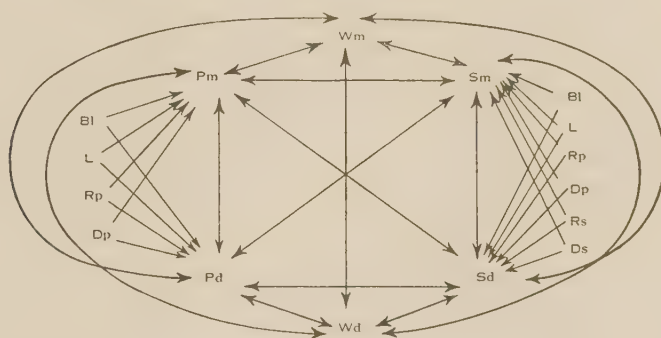


Fig. 1.—Diagrammatic representation of the complete experiment in which double-headed arrows indicate possible correlations in the experimental material. The computed correlations are given in Table 1.

the time of testing, body weight (*Wm* for dams, *Wd* for daughters) was only determined once during the course of the experiment. The dependent variables are four in number: primary response of dams (*Pm*), primary response of daughters (*Pd*), secondary response of dams (*Sm*), and secondary response of daughters (*Sd*).

III. RESULTS

The statistical analysis of the data falls into two parts: (1) the effect of the independent variables (treatments and body weight) on both primary and secondary response, and (2) the estimation of genetic components of variation (h^2) for primary and secondary response, and the genetic covariances between primary and secondary responses. The complete experiment is represented diagrammatically in Figure 1 in which the double-headed arrows indicate possible correlations in the experimental material. Possible effects of the independent variables are represented by single-headed arrows. A standard analysis of variance and covariance (Fisher 1954) is made of the body weights and primary responses of the daughters and dams. The error term of this analysis enables estimates to be made of the correlations between these variables (see Table 2). Since both dam and daughter have been placed in the same experimental unit, the analysis of variance cannot be directly extended to

study any differences in the mean response of daughters compared with dams or any differential response of them to the treatments. The sum of the responses of the dams and daughters in each experimental unit is a measure of the joint response of both individuals while the difference between them should be zero (on the average) if the animals are responding similarly to treatment. The variance of a sum or a difference may be directly computed for the variances and covariances of this variable (cf. Rao 1952, p. 241), thus enabling the analyses to be completed.

TABLE 2
CORRELATIONS WITHIN THE EXPERIMENTAL MATERIAL
For definition of symbols, see text

Correlation Coefficient	<i>Sm</i>	<i>Sd</i>	<i>Pm</i>	<i>Pd</i>	<i>Wm</i>	<i>Wd</i>	
r_{93}	1	-0.037 1	0.087 0.179	-0.018 0.176	0.040 0.128	0.094 -0.031	<i>Sm</i> <i>Sd</i>
r_{108}			1	0.209* 1	0.065 0.101 1	-0.313** -0.256** 0.018 1	<i>Pm</i> <i>Pd</i> <i>Wm</i> <i>Wd</i>

* $0.05 > P > 0.01$.

** $0.01 > P > 0.001$.

The analysis of variance of the sum of the daughter and dam primary responses indicates large block differences, thus confirming previous work on which the experiment was designed. Both daughter and dam responses are very strongly dependent on dose, and significance of the daughter-dam difference mean square shows the dose-response slope to be a function of age, since the daughters and dams differ alone in this respect. While the average effect of route of injection is not significant, the highly significant interaction of this variable with lines demonstrates that the intraperitoneal route led to higher response in the high-sensitivity line and a lower response in the low-sensitivity line, whereas the lines do not differ with intravenous route of administration. The remaining significant item in the analysis of variance table (Table 3) is the interaction of blocks with lines for the daughter-dam difference. This finding is difficult to explain and may be due either to non-additivity of the responses over blocks (although if this were serious other interactions should be significant) or chance. Having removed variance and covariance due to all known sources of variation the remainder in the "error" term may be used to calculate heritability.

From Table 3 it is apparent ($\chi^2_{(L)} = 0.004$, $0.05 < P < 0.1$) that the variances of the primary response of daughters and dams are not significantly different. In this case the correlation coefficient gives an estimate of heritability $h^2(\%) = 200r$. Therefore the heritability of primary response is 41.8 ± 19.4 per cent. where the standard error follows the $\pm$ sign.

The analysis of variance of the secondary responses follows the same pattern as that of the primary responses. Further correlation coefficients may be determined,

however, since both secondary and primary response have been determined in the same individuals. These correlation coefficients complete Table 2. Since none of

TABLE 3

ANALYSIS OF VARIANCE AND COVARIANCE OF PRIMARY RESPONSES CORRECTED FOR BODY WEIGHT
For definition of symbols, see text

Source of Variation	D.F.	<i>PmPm</i>	<i>PmPd</i>	<i>PdPd</i>	$(Pm + Pd)^2$	$(Pm - Pd)^2$
<i>Bl</i>	3	23,802	15,237	10,145	64,421***	3,473
<i>L</i>	1	29	-222	1,672	1,257	2,145
<i>Dp</i>	1	44,258	31,294	22,127	128,973***	3,797*
<i>Rp</i>	1	60	-139	316	98	654
<i>Bl</i> × <i>L</i>	3	6,142	-919	140	4,444	8,120*
<i>Bl</i> × <i>Dp</i>	3	4,438	-796	747	3,593	6,777
<i>Bl</i> × <i>Rp</i>	3	2,238	-148	640	2,582	3,174
<i>L</i> × <i>Dp</i>	1	3	33	439	508	376
<i>L</i> × <i>Rp</i>	1	1,648	3,181	6,139	14,149***	1,425
<i>Dp</i> × <i>Rp</i>	1	606	804	1,068	3,282	66
Error	107	51,046	7,571	50,362	116,500	86,266

* $0.05 > P > 0.01$. ** $0.01 > P > 0.001$. *** $P < 0.001$.

the additional coefficients appear significant, little will be gained by correction of secondary responses for this concomitant variation in the analysis of variance (see

TABLE 4

ANALYSIS OF VARIANCE AND COVARIANCE OF THE SECONDARY RESPONSES

The grouped interactions were tested separately and none found significant. For definition of symbols, see text

Source of Variation	D.F.	<i>SmSm</i>	<i>SmSd</i>	<i>SdSd</i>	$(Sm + Sd)^2$	$(Sm - Sd)^2$
<i>Bl</i>	3	4,340	6,630	13,677	31,277***	4,757***
<i>L</i>	1	1,041	1,540	2,278	6,399**	239
<i>Dp</i>	1	1,333	2,220	3,698	9,471***	591
<i>Rp</i>	1	3,190	4,443	6,188	18,264***	492
<i>Ds</i>	1	5,113	3,274	2,096	13,757***	661
<i>Rs</i>	1	410	455	504	1,824	4
<i>Bl</i> × <i>L</i>	3	1,576	1,490	1,739	6,295*	335
<i>Bl</i> × <i>Dp</i>	3	1,638	1,345	1,155	5,483*	103
<i>Bl</i> × <i>Rp</i>	3	1,073	-986	6,057	5,158*	9,102**
<i>Bl</i> × <i>Ds</i>	3	3,515	-1,764	1,096	1,083	8,139**
<i>Bl</i> × <i>Rs</i>	3	759	648	1,146	3,201	609
Other first-order interactions	10	2,678	-42	2,994	5,588	5,756
Error	94	22,668	-978	31,166	51,878	55,790

* $0.05 > P > 0.01$. ** $0.01 > P > 0.001$. *** $P < 0.001$.

Table 4), which is presented in the same form as that for primary responses. Significant time trends occurred during the course of the experiment as evinced by the

block effect. That these trends were not constant over both dams and daughters is shown by significance of the block effect in the daughter-dam difference analysis. Further time trends of a more complex nature are seen in the interaction terms of the analysis of variance. This indicates that the various treatment effects were not of the same magnitude over the whole experiment.

It is clear that the variances of the secondary responses of the dams and daughters are not significantly different ($\chi^2_{(L)}=2.63$, $0.1 < P < 0.2$). Comparison of the mean variances of the primary responses with that of the secondary responses indicates that the latter are considerably less variable than the former ($\chi^2_{(L)}=12.77$, $P < 0.001$). As above, we may estimate the heritability of secondary response from the correlation coefficient given in Table 2, and $h^2 = -7.4 \pm 20.7$ per cent. based on figures in the "error" term.

The correlation between the primary response of dams and the secondary response of daughter or vice versa is a measure of the *genetic determination* of secondary response by primary response. The correlation between primary and secondary response in the same individual obviously includes environmental factors as well as genetic. It is obvious, however, that none of these correlations are of any significance in our material (Table 2).

IV. DISCUSSION

This paper illustrates a convenient way of obtaining an estimate of heritability while at the same time examining other causes of variation. The accuracy of the method in a given situation is determined by the number of experimental units which are employed. A number of estimates obtained in a series of experiments of this kind may be combined to give estimates of greater precision. Estimates of heritability from controlled experiments must be interpreted cautiously since the value obtained is to some extent under the control of the experimenter. Heritability is defined as the percentage of the total variance of a character taken up by the additive genetic variance. By means of planned experimentation certain components of variance such as the effect of time, age, dose, route of administration, and additive genetic variance, etc. can be separately determined. Depending on the definition of total variance, different estimates of heritability are obtained, indicating the arbitrary nature of this concept. In comparing different estimates care must therefore be taken that estimates are obtained in similar circumstances. The estimates of heritability given above are maximal or approaching this since all known components have been removed from the total variance leaving only experimental error and additive genetic variance. It would be expected, therefore, that in the field, under less controlled conditions, the heritability of response to this complex antigen will be very small. (From Table 3 additive genetic variance is $[4 \times (7571)^2]/51046 = 4492$ which is comparable with other mean squares for the primary responses of the mothers or daughters.) Under the less-controlled conditions of the first paper of this series the daughter-dam correlation of primary response ($h^2 = 11.0 \pm 11.7$ per cent.) was not found significant for this reason, although roughly three times the number of animals were used in the determination.

The various block differences found with both primary and secondary response are indicative of secular changes in both the level of response and in the magnitude of differences induced by some treatments. Findings of this nature are common in other biological material, for example the secular changes in median effective dose and dose-response line slope reported by Biggers (1953) with reference to the Allen-Doisy oestrogen-assay technique. The findings emphasize the need for all comparisons to be made at the same time, and, if an experiment is so large that this is impracticable, an appropriate block replicated experimental design used.

With primary response (Table 3) there is no difference between the lines in overall response, but examination of the route-line interaction shows that while both lines react equally to intravenous injection, with intraperitoneal injection the high oestrogen-selection line gives a higher response than the low oestrogen-selection line. Whether this effect is due to genetic correlation of primary response with oestrogen-sensitivity or due to genetic drift cannot be ascertained from the present data. Irrespective of route of administration there is a significant difference between the lines in their secondary response, with the low oestrogen-sensitivity line having a higher response than the other line. Taken together with the absence of significant correlation between primary response and secondary response this difference between the responses strengthens the view that the factors governing the response of an animal to a priming dose of a complex antigen are considerably different from those governing response to a subsequent encounter with that antigen. Further evidence in support of this view is that the variability of primary responses is about twice that of secondary responses.

It was found that the route of injection of the priming dose affected the response to a secondary dose of antigen in that those primed by the intravenous route responded more to a secondary dose than those primed by the intraperitoneal route, suggesting that this antigen when administered intravenously impresses itself more permanently, or strongly, upon the antibody-forming mechanism than when administered intraperitoneally.

V. ACKNOWLEDGMENTS

We wish to thank Professor C. W. Emmens and Dr. J. M. Rendel for their interest in the investigations and for criticism of the manuscript.

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PATHOLOGY OF INFESTATION OF THE RAT WITH *NIPPOSTRONGYLUS MURIS* (YOKOGAWA)

I. CHANGES IN THE WATER CONTENT, DRY WEIGHT, AND TISSUES OF THE SMALL INTESTINE

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[Manuscript received December 18, 1956]

Summary

The fresh weight of the rat's small intestine together with its contents, as well as the intestinal tissue alone, was doubled during the 15 days following infestation by the nematode *N. muris* (Yokogawa, 1920). This increase, which was independent of the growth of the rat, was largely due to a two-fold increase in the water content of both the lumen and the tissues. The dry weight of the tissues was increased by half.

Associated with these changes was a two-fold increase in the tangential diameter of the jejunum which was the site of the infestation. This was not due solely to a dilatation but also to the increase in the amount of intestinal tissue. The width of the circular layer of the muscularis externa of the jejunum was doubled and the longitudinal layer was increased by half. Histologically, the increase of the circular layer appeared to be due to hypertrophy, but an increase in the number of the smooth-muscle nuclei suggested that hyperplasia might also be present.

I. INTRODUCTION

The gross and microscopic pathology of the small intestine of rats infested by the nematode *Nippostrongylus muris* (Yokogawa, 1920) has been described by Porter (1935) and Taliaferro and Sarles (1939). The latter workers reported that the jejunum may be dilated to two or three times its normal diameter, and that the dilatation was associated with an accumulation of fluid and gas. No mention was made in either of these papers of an apparent hypertrophy of the tissues of the infested jejunum.

Experiments were done to show the distribution of water and dry matter between the contents and tissues of the infested small intestine, and to determine the changes occurring in these relationships during the course of the infestation which persists for about 2 weeks after the subcutaneous introduction of infective larvae (Africa 1931). Measurements were made of the tangential diameters of the jejunum and of the thickness of the muscularis externa because any quantitative changes in the tissues would affect the weights of water and solid matter. The number of nuclei in sections of the circular layer of the muscularis externa were estimated to determine whether hyperplasia was present.

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II. METHODS

(a) *Water and Dry Matter Content*

(i) *Changes in the Small Intestine together with its Contents.*—This experiment was planned to measure the water content and dry weight of the intact small intestine daily during the 15 days of infestation. Sixteen groups of five female albino rats were randomly allotted to days 0–15. The groups representing days 1–15 were infested by a subcutaneous injection of 3500–4000 infective larvae of *N. muris*. Four rats from each group were killed in an ether jar after a fast of about 6 hr. Some ingesta remained in the small intestine after this fast, and undoubtedly affected the intestinal weights recorded. On the other hand, a fast for as long as 24 hr failed to empty completely the small intestine and may have affected the volume of water in the lumen. The intact small intestine, which was separated rapidly from the stomach, caecum, and mesentery, was lightly blotted and weighed in a covered petri dish. It was then dried to constant weight at about 100°C.

The severity of the infestations were judged subjectively. No worm counts were possible as the intestinal contents were not removed.

(ii) *Changes in the Small-intestinal Tissue after Removal of the Contents.*—Preliminary examination of the results of the first experiment indicated that groups of five rats killed at intervals of 3 days would be sufficient to provide the information required. Accordingly, six groups to represent days 0, 3, 6, 9, 12, and 15 were used. The small intestine, after its separation from the other organs, was slit open longitudinally, the contents were lightly scraped off, and the mucosa lightly blotted. Weighing and drying were carried out as before.

In each experiment the data were corrected to the mean body weight at death, on the grounds that a linear relationship exists between intestinal weight and body weight in the growing rat (Brody 1945).

(b) *Measurements of the Diameter of the Jejunum and the Width of the Muscularis Externa*

Eighteen female albino rats weighing from 100 to 150 g were paired according to weight. One of each pair was randomly selected for infestation by the subcutaneous injection of about 4000 infective larvae. The pairs were then randomly divided between three groups. There were, therefore, three infested and three non-infested rats in each group. One group was killed 7 days after infestation, the second after 10 days, and the third after 14 days. By the end of the first week after skin penetration the parasites had been in the small intestine for 4 or 5 days (Yokogawa 1922). The tenth day was chosen because Taliaferro and Sarles (1939) reported that the most marked histological reactions of the host occur about that time, and the fourteenth day chosen because Africa (1931) showed that the infestation begins to decline at about the end of the second week.

The rats were killed in an ether jar. The small intestine was removed, a specimen about 1 in. in length was taken from the jejunum about 6 in. below the pylorus, and fixed in corrosive formalin so as to reduce shrinkage to a minimum.

Care was taken to ensure that the pairs of sections, each section about $\frac{1}{2}$ in. apart, subsequently cut from these specimens, were at right angles to the longitudinal axis of the gut. One pair of sections from each rat was stained with haematoxylin and eosin and the other with celestine blue and eosin to differentiate the nuclei.

Because the sections, particularly those taken from the infested rats, were not usually circular after fixation, a comparison of diameters was made by measuring two tangential diameters perpendicular to each other. The initial diameter was chosen in a random manner. A grid in an ocular of the microscope was used to measure the diameters of a pair of sections from each rat.

The widths of the circular and longitudinal layers of the muscularis externa were measured with an ocular micrometer. Four measurements approximately 90° apart were made on each section. Pairs of sections were again used; thus eight measurements were made on the jejunum of each rat.

The number of nuclei in the circular layer was estimated to determine if hyperplasia was present. A radial grid of eight divisions was made on a photographic negative, and the number of nuclei in four divisions was counted when the grid was centrally placed over the section with a drop of immersion oil between the grid and the cover-slip. The actual divisions counted were decided for each section by random numbers. This random distribution was not always possible as occasionally a tear in the muscle layers meant that a particular division had to be abandoned for another. Counts were made on pairs of sections from each rat.

III. RESULTS

(a) *Water and Dry Matter Content*

The infestations, as judged by the size and congestion of the small intestines, were moderate rather than heavy. In the early stages of the intestinal phase the apparent dilatation, which appeared on about the fifth day, was confined to the upper jejunum, and was well defined at both its proximal and distal ends. The infested section was markedly congested, and the associated blood vessels in the mesentery were engorged. Although there may have been an increase in the water content of the ileum at this stage, this section of the bowel was not obviously distended until about the eighth day after the injection of the larvae. The increased diameter of the ileum which, however, was never as pronounced as that of the upper jejunum, appeared to be solely due to a mechanical distension by fluid, whereas that of the jejunum itself was also associated with a marked thickening of the intestinal tissue. Some time after the tenth day the dilatation began to disappear. Towards the fifteenth day the jejunal wall appeared flabby and thickened rather than distended. The mucosal surface of the intestine, particularly that of the jejunum, felt rough when lightly stroked by a scalpel blade.

(i) *The Intact Intestine*.—In Figure 1 (line A) are shown the corrected mean fresh weights of the small intestines at daily intervals. In Figure 2 are shown the corrected mean water and dry matter weights, also at daily intervals. In both instances each point represents the mean of four rats.

If orthogonal polynomials are fitted to the data for water content, the quadratic curve is significant at the 5 per cent. level, but not at the 0.1 per cent. level. This

curve suggests that the water content reached a maximum on about the thirteenth to fourteenth day after infestation by injection. On the other hand, the linear increase of both the dry matter and fresh weight was significant at the 0.1 per cent. level, whereas the quadratic component was non-significant. These fitted curves

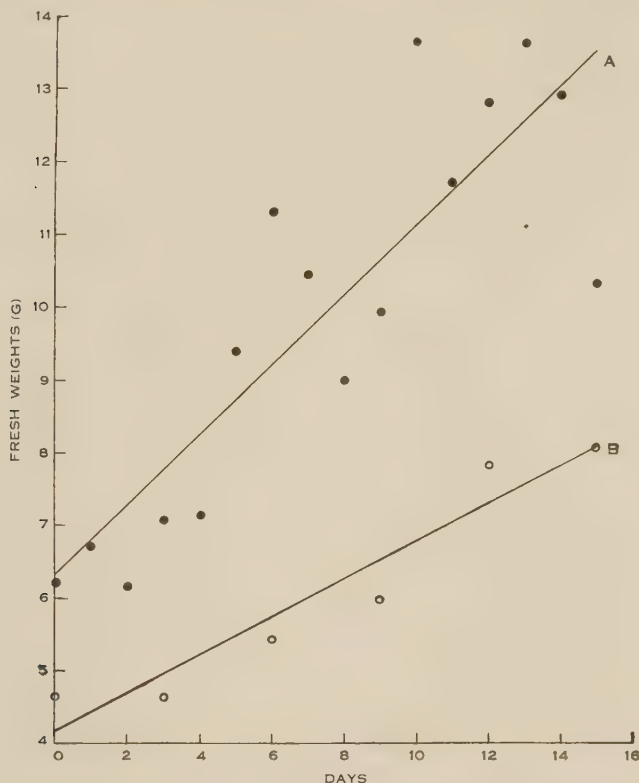


Fig. 1.—Changes in the fresh weights of the rat's small intestine. ● Daily fresh weights of the small intestine together with its contents. Each point represents the mean of four rats. ○ The fresh weights at 3-day intervals of the small intestine from which the contents had been removed. Each point represents the mean of five rats. Line A, intact intestine. Regression fresh weight on time: $b = 0.4798 \pm 0.0776$ ($P < 0.001$). Line B, intestinal tissue. Regression fresh weight on time: $b = 0.7892 \pm 0.1220$ ($P < 0.05$).

should, however, be accepted with caution as the variability of the observations does not allow a more precise determination of the relationship of time with the changes that occurred throughout the infestation.

On the other hand, if the linear regressions of the fresh weight, water, and dry matter content on time be taken as a measure of the changes, there were increases which were all significant at the 0.1 per cent. level. These regression coefficients are given in the legends to Figures 1 and 2. From Figure 1 it can be seen that the mean fresh weight of the small intestine increased two-fold over the duration of the experiment. Similarly, from Figure 2, it is apparent that the water content was slightly more than doubled, whereas the dry matter was increased by half.

(ii) *Tissue of Small Intestine*.—The change, at intervals of 3 days, of the fresh weight of the small intestine from which the contents were removed is shown in Figure 1 (line B). The changes in water and dry matter content are shown in Figure 3. Each point represents the mean of five rats.

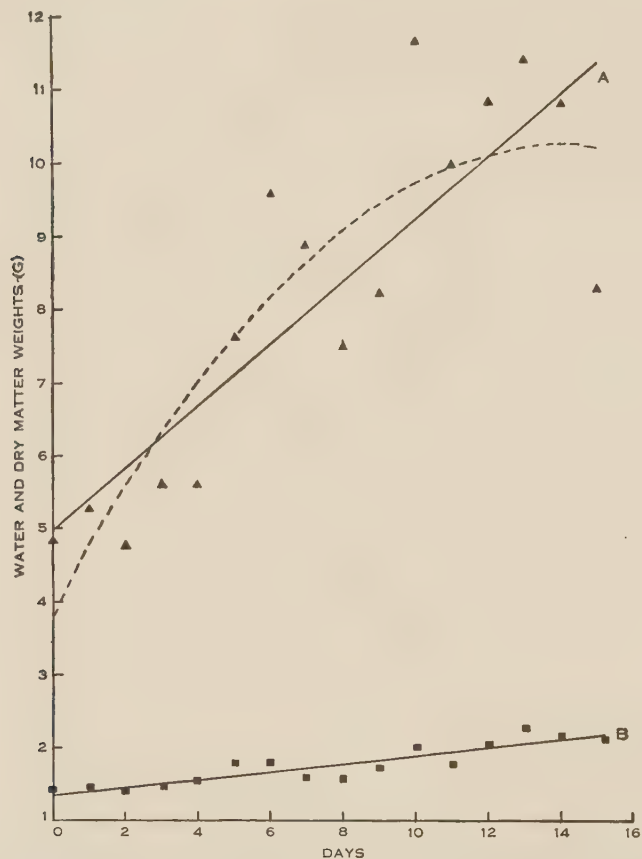


Fig. 2.—Daily water and dry matter weights of the rat's small intestine together with its contents. Each point represents the mean of four rats. ▲ Water content. ■ Dry matter. Line A, regression water content on time: $b=0.4252 \pm 0.0742$ ($P<0.001$). - - - Quadratic curve ($P<0.05$). Line B, regression dry matter on time: $b=0.0546 \pm 0.0069$ ($P<0.001$).

If orthogonal polynomials are fitted there appears to be a linear increase in each instance, all significant at the 5 per cent. level, but the significance of these curves should also be accepted with caution. As before, the linear regression coefficients of the weights on time are shown in the legends to Figures 1 and 3. They are significant at the 5 per cent. level. Again, there was a two-fold increase in the weight of the fresh intestine; the water content was slightly more than doubled and the dry matter increased by half.

(b) *Measurements of the Diameter of the Jejunum and the Width of the Muscularis Externa*

(i) *General*.—In Plate 1, Figures 1 and 2, are photographs of cross sections of typical normal and infested jejunum respectively. The cellular changes during an initial infestation have been described in detail by Taliaferro and Sarles (1939). Attention must be drawn, however, to the apparent increase in the bulk of the mucosa of the infested intestine. This did not appear to be due solely to the oedematous conditions of the irregularly shaped villi which replaced the regular finger-like villi of the normal animal.

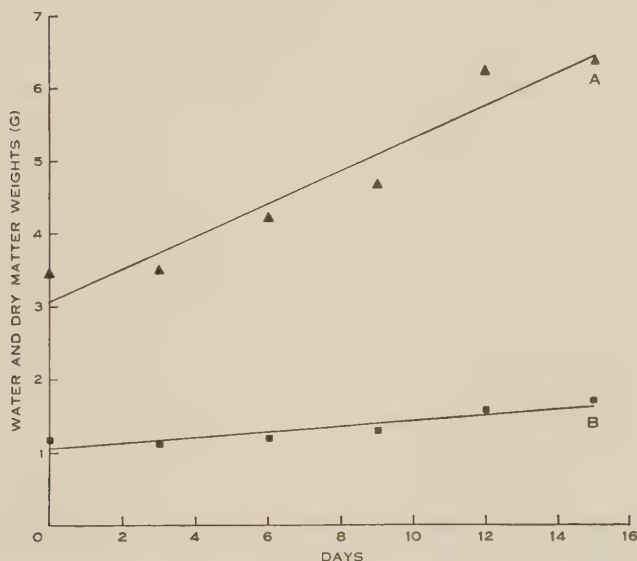


Fig. 3.—Water and dry matter weights at 3-day intervals of the rat's small intestine from which the contents had been removed. Each point represents the mean of five rats. ▲ Water content. ■ Dry matter. Line A, regression water content on time: $b=0.6737 \pm 0.0986$ ($P<0.05$). Line B, regression dry matter on time: $b=0.1154 \pm 0.0262$ ($P<0.05$).

(ii) *Tangential Diameters*.—The mean tangential diameters of all the infested intestines together was 4.408 mm (± 1.162), whereas the mean of the non-infested rats was 2.266 mm (± 0.440). This difference was significant at the 0.1 per cent. level. The diameter of the jejunum in the infested rat was, therefore, almost doubled under the conditions of this experiment. The comparative sizes of a typical infested and non-infested jejunum are shown in Plate 1, Figures 1 and 2.

(iii) *Width of the Muscularis Externa*.—In Table 1 are shown the mean widths of the circular and longitudinal layers of the muscularis externa in both infested and non-infested rats. The mean width of the circular layer, when all the measurements of the infested rats were taken together, was 156.2μ . This was significantly greater at the 0.1 per cent. level than the mean width of the circular layer in the

non-infested rats, which was 76.0μ . Similarly the mean width of 71.5μ for the longitudinal layer in the infested rats was significantly greater at the 5 per cent. level than the mean width of 45.7μ in the non-infested rats. It can be seen, therefore, that the circular layer of the muscularis externa was doubled in width whereas the longitudinal layer was increased by nearly half.

There was insufficient evidence to determine if the rate of increase in the width of the muscularis externa was greater before the seventh day or between the seventh and tenth days, but there was little change after the tenth day.

Plate 1, Figures 1 and 2, provides a comparison of the widths of the complete muscularis externa in the infested and non-infested rat. In Plate 2, Figures 1 and 2, are compared the more detailed appearances of the circular layer of this muscle.

TABLE 1

MEAN WIDTHS OF THE MUSCULARIS EXTERNA IN JEJUNAL SECTIONS FROM INFESTED AND NON-INFESTED RATS

Day after Infestation	Width of Circular Layer (μ)		Width of Longitudinal Layer (μ)	
	Infested	Non-infested	Infested	Non-infested
7	110.9	68.6	63.6	39.5
10	173.5	82.2	66.7	53.6
14	184.4	77.2	84.0	44.0
All rats	156.2	76.0	71.5	45.7

The magnification is the same in both figures. It can be seen that the muscle in Plate 2, Figure 2, increased in width due to a thickening of the individual fibres. The nuclei, which are less densely stained, are also thicker in cross section, and possibly shorter. This appearance is similar to the hypertrophy of cardiac muscle as described by Wright (1951).

(iv) *Number of Nuclei in the Circular Muscle.*—The estimated mean number of the nuclei of the smooth muscle in one division ($\frac{1}{8}$ of the cross section) of the circular layer of the muscularis externa of the jejunum of infested rats was 241, and in the non-infested rats the number was 175. The 95 per cent. confidence limits were within the range of 262–221 and 188–161 respectively. This difference was significant at the 5 per cent. level, and appears to indicate that part at least of the increase in width of this layer was due to hyperplasia. It was noticed, however, that the number of nuclei was markedly increased in those areas of the sections where the fibres were contracted, and that these contracted areas occurred more frequently in the sections from infested rats. Mitotic figures were not seen.

IV. DISCUSSION

The effect of growth on intestinal weight during the 15 days of infestation in both water and dry matter content experiments can be ignored because all weights were adjusted to the mean body weight at death.

The marked gains in the fresh weights of the intact intestine and intestinal tissue alone were associated with increases in both water and dry matter content. It was not possible, however, to make a precise statement relating these changes to the course of the infestation. They appeared to be linear with time from day 0, except that if the quadratic curve of Figure 2 can be accepted as truly descriptive, the water content of the intact intestine may have reached a peak on about the thirteenth or fourteenth day, and thereafter declined. From observation of many infested rats, a decline in water content, and probably also of fresh weight, was expected at the end of 2 weeks, but this was not definitely established by these experiments. Furthermore, it seems unlikely that there would be a linear increase of weights over the first 2 or 3 days. The parasites do not begin to enter the small intestine until about 48 hr after the subcutaneous injection of the larvae because there is an intervening passage through the lungs. It seems probable that a precise curve relating weight changes to time would be sigmoid in form. There is, in fact, a suggestion in both Figures 1 and 2 that the fresh weight and water content of the intact intestine did not begin to increase until the third or fourth day, but there were insufficient data to confirm this.

The increase in the fresh weight of the intact intestine was not due to the greater retention of ingesta as the infestation progressed, since there was also a progressive increase in the weight of the intestinal tissue alone. It is possible, however, that the greater scatter of the daily mean weights in the experiment concerned with the intact intestine was due in part to variations in the amount of retained ingesta. The weight of the parasites was ignored because of their small size.

It is clear that, since the water content of both the intact intestine and of intestinal tissue alone was slightly more than doubled whereas the dry matter increased by only half, the former was largely responsible for the gain in fresh weight. The increase in water content was not confined solely to the intestinal tissue since it was observed to accumulate in the lumen. This is confirmed in Figures 2 and 3 from which it can be estimated that there was an increase of about 6.5 g of water in the intact intestine, and about 3.5 g in the intestinal tissue. The subsequent histological examination of the jejunum confirmed the accumulation of water in the tissues, which was largely confined to an oedema of the mucosa. The submucosa and the muscularis externa did not appear to be oedematous, although any increase in the absolute amount of tissue would account for some of the increase in water content.

The increase in size of the muscularis externa undoubtedly accounted for some of the gain in dry matter of the intestinal tissue. The circular layer of this intestinal muscle was doubled in thickness, whereas the longitudinal layer increased by half. No attempt was made to determine quantitatively if there was an absolute increase in the amount of mucosal tissue although examination of sections such as those shown in Plate 1, Figure 2, suggested that there was an increase.

Histologically the changes in the circular muscle were consistent with hypertrophy. Although the evidence for hyperplasia of this muscle was doubtful, it was difficult to account for the apparent increase in the mucosal tissue by any other

means. It was also difficult, however, to determine if there was a difference between the density of mitotic figures in the epithelial cells of the normal and infested mucosa.

The enlargement of the infested jejunum was, therefore, not due solely to a dilatation by fluid or gas, but, in part at least, to an increase in the bulk of tissue. Had a dilatation been solely responsible, the intestinal tissue would have been thinner. On the other hand, the accumulation of fluid in the lumen must have been partly responsible for the increase in size since, in the terminal stages of an infestation, when the water was leaving the gut, it was thick and flabby rather than distended. Damage to the epithelium may have accounted for the accumulation of the fluid in the lumen of the gut, but further work is required to determine if a more active process was involved.

The undoubted hypertrophy of the muscularis externa together with the possible increase in the mucosal tissue suggested hyperactivity of the gut. Whether the presence of the parasites or the fluid in the lumen or both, stimulated this activity cannot be decided at this stage. There is, however, some evidence that the parasites themselves are the primary cause, for in the lightly infested rat they were collected together in reddened and thickened pockets which bulged outwards from the serous surface of the jejunum. Work has been planned to investigate changes in muscular activity during the infestation.

V. ACKNOWLEDGMENTS

The author is indebted to Mr. V. J. Bofinger, formerly of the Division of Mathematical Statistics, C.S.I.R.O., for assistance in planning the experiments, and for some of the statistical analyses, and to Mr. A. M. W. Verhagen of the same Division for further analyses.

Thanks are also due to Mr. I. T. Roper who took the photographs, and to Miss A. M. Bartle who drew the graphs.

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EXPLANATION OF PLATES 1 AND 2

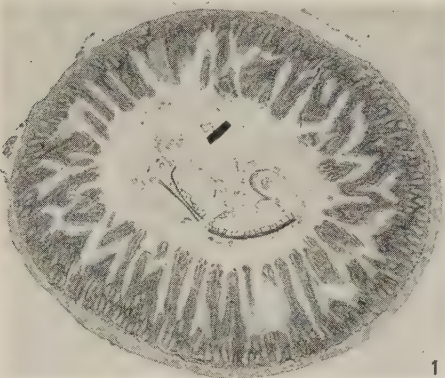
PLATE 1

Dilatation of the jejunum

Fig. 1.—Photograph of a cross section of the jejunum of a normal rat.

Fig. 2.—Photograph of a cross section of the jejunum of a rat infested by the nematode *N. muris*. The size has been increased. The villi are more irregularly shaped and are oedematous. The muscularis externa is thickened. The break in the integrity of the intestinal wall at the lower left-hand corner is probably due to the penetration of a parasite.

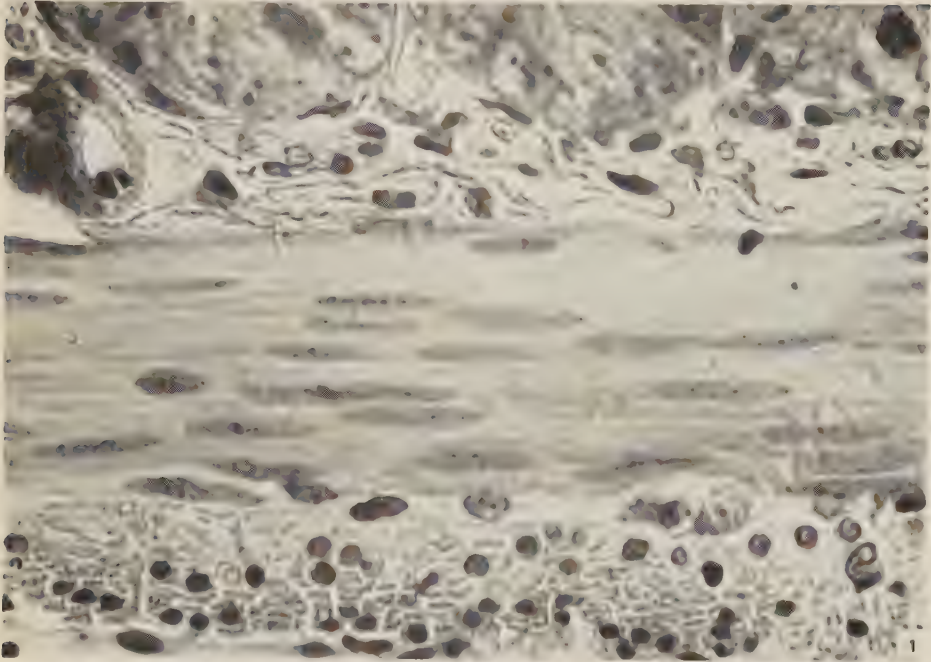
PATHOLOGY OF NIPPOSTRONGYLOSIS OF THE RAT. I



1 MM



PATHOLOGY OF NIPPOSTRONGYLOSIS OF THE RAT. I



50 μ



PLATE 2

The circular layer of the muscularis externa

- Fig. 1.—Photomicrograph of a section of the circular layer of the muscularis externa of the jejunum of a normal rat.
- Fig. 2.—Photomicrograph of a section of the circular layer of the muscularis externa of the jejunum of a rat infested by the nematode *N. muris*. The width of the muscle has been increased due to a thickening of the individual fibres. The nuclei which are less densely stained, are also thicker in cross section.

THE AMINO ACID COMPOSITION OF HYDROLYSATES OF MICROBIAL PREPARATIONS FROM THE RUMEN OF SHEEP

By R. A. WELLER*

[*Manuscript received December 21, 1956*]

Summary

Samples of bacteria and of protozoa were separated from the rumen fluids of sheep which had been fed four different types of ration. Amino acid analyses by ion-exchange chromatography were performed on hydrolysates of "whole protein" preparations of the microbial fractions.

The composition of the bacterial hydrolysates was remarkably uniform, and the amino acid distribution was similar to those reported for pasture leaf proteins. Compared with the bacteria, the protozoa appeared richer in "essential" amino acids, particularly lysine.

I. INTRODUCTION

In the feeding of ruminants, estimates of the quantity and quality of protein intake are complicated by the effects of microbial activity in the rumen. The mixture of proteins reaching the abomasum contains variable proportions of dietary and of microorganism proteins, the latter having been synthesized in the rumen from nitrogen sources provided principally by the proteins and other nitrogenous compounds of the diet.

Evidence of the extent of this conversion and its significance has been reviewed by Chalmers and Synge (1954) and McDonald (1954). The nutritive values of rumen bacterial proteins and rumen protozoal proteins have been examined in a number of feeding experiments with monogastric animals (see McNaught *et al.* 1954), but little information of the quantitative amino acid composition of these microbial proteins is available. Holmes, Moir, and Underwood (1953), using paper chromatography analysed bacterial preparations from the rumen fluids of "dry" and "green"-fed sheep. The microbiological analyses by Duncan *et al.* (1953) of hydrolysates of rumen digesta from calves also give an approximate picture of the partial amino acid composition of the microbial protein mixture, as the host animals had received an essentially protein free diet.

In the present investigations bacterial and protozoal fractions from the rumen liquids of sheep which had been fed four different types of ration were examined. Amino acid analyses of hydrolysates of "whole protein" preparations of the isolated microbial fractions were made by the Moore and Stein (1951) method of chromatography on "Dowex 50" ion-exchange resin columns.

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II. MATERIALS AND METHODS

(a) *Collection of Rumen Liquids*

The following diets were fed to sheep for periods of at least 3 weeks prior to collecting rumen liquids:

Diet 1.—Wheaten hay chaff (0.89 per cent. nitrogen)—*ad lib.*

Diet 2.—Lucerne hay chaff (2.91 per cent. nitrogen)—*ad lib.*

Diet 3.—Wheaten straw (600 g) providing 2.3 g nitrogen daily, crushed oat grain (200 g) providing 2.9 g nitrogen daily, and urea (15 g) providing 7.0 g nitrogen daily.

Diet 4.—Mixed green pasture—grazing animals.

In the first three trials, samples of rumen liquid were withdrawn from two sheep through permanent fistulae 2 hr after offering fresh fodder. The fourth sample was obtained by filtering the rumen contents of three slaughtered animals through muslin gauze.

(b) *Separation of Microbial Fractions*

After filtering the rumen liquid through the fine muslin gauze it was centrifuged at low speed for the minimum time necessary to deposit all protozoa as determined by microscopic examination of the supernatant liquid. The protozoal sludge was washed free of bacteria by repeatedly resuspending in water and slow centrifuging until the washings were quite clear. Microscopic examination of the residue revealed almost complete freedom from contamination by either plant particles or bacteria. The combined supernatant liquids were diluted, filtered through No. 1 Whatman paper, and the bulk of the bacteria deposited in a Sharples super centrifuge operating at 25,000 r.p.m. The product appeared almost free from plant material and protozoa. Whole proteins were prepared directly from the bacterial and the protozoal sludges using the modified method of Lugg and Weller (1944).

The rumen fluid from the pasture-fed sheep contained a considerable quantity of plant material and a variation in procedure was necessary. On slow centrifuging a layer of dark green slime was deposited above the protozoal layer. The speed and time of centrifuging used in depositing the protozoa from the other rumen fluids was adopted, and the washing repeated until the green layer was no longer evident. Only the supernatant liquid from the initial centrifuging was used for the separation of bacteria.

In all of these separations emphasis was placed on obtaining relatively pure bacterial and protozoal fractions at the expense of quantitative yields and complete representative samples. Only those bacteria free floating in the rumen fluid and large enough to be deposited in the Sharples centrifuge were included, and in the case of the samples from diet 4 there would have been considerable losses of the larger bacteria and smaller protozoa.

(c) *Nitrogen Content of Preparations*

The results of nitrogen determinations on the dry whole proteins by the Kjeldahl method are shown in Table 1.

(d) Amino Acid Analyses

Samples of whole protein containing about 15 mg total nitrogen were refluxed for 24 hr with 100 ml of twice glass-distilled constant-boiling HCl. The hydrolysates were concentrated under reduced pressure at 40°C to a volume of 4 ml, diluted to 50 ml, and centrifuged. Aliquots containing 0.3 mg total nitrogen were taken for the chromatography of acidic plus neutral amino acids and 0.6 mg total nitrogen for the basic amino acids.

Following the procedure of Moore and Stein (1951) acidic and neutral amino acids were separated on a 100-cm column of 8 per cent. cross-linked "Dowex 50" resin and the basic amino acids on a 15-cm column. The procedure as outlined for the operation of the columns was followed in every detail except for the use of pH 3.33 citrate in the elution of acidic amino acids and the omission of detergent from all

TABLE 1
PERCENTAGE NITROGEN IN DRY, MICROBIAL WHOLE PROTEINS

	Diet 1	Diet 2	Diet 3	Diet 4
Bacteria	9.3	11.1	10.4	12.4
Protozoa	3.8	6.5	2.7	7.9

eluting buffers. For the determination of amino acids in the effluent fractions the photometric ninhydrin method of Moore and Stein (1948) was used, substituting ethanol for propanol in the diluent solution. The sequence of peaks in the elution curves corresponded with the pattern illustrated by Moore and Stein (1951) and the identity of the individual components was confirmed by the chromatography of pure amino acids.

A new type of fraction cutter* was used which consisted essentially of an automatically operated blowout pipette, silicone-coated internally. With this device, fraction sizes varied by less than 0.5 per cent. and the absence of intermixing between consecutive samples contributed to a high degree of resolution with even the difficultly separable amino acids. Compared with the Moore and Stein (1951) elution curve improved separation was obtained between isoleucine and leucine and between tyrosine and phenylalanine but proline was poorly separated from glutamic acid. For the determination of proline a separate sample of hydrolysate was chromatographed and proline estimated by the method of Chinard (1952). With each of the bacterial hydrolysates a small peak, probably due to diaminopimelic acid, appeared immediately before the methionine peak but there was no other evidence of measurable quantities of ninhydrin-positive substances of doubtful identity with any of the microbial hydrolysates. The small amount of cystine present did not give an obvious peak and this amino acid was determined separately. A separate sample of whole protein was used and cystine oxidized to cysteic acid by the method of Schram, Moore,

* The fraction cutter and turntable assembly were designed in this Laboratory by Mr. V. A. Stephen and a detailed description of the apparatus will be published elsewhere.

and Bigwood (1954). After hydrolysis and elution through the 100-cm "Dowex 50" column, the cysteic acid yielded a sharp peak in a position where no ninhydrin-positive substance had been detected with the hydrolysate from the unoxidized sample.

The results expressing amino acid nitrogen as percentages of the total nitrogen of the humin-free hydrolysates are set out in Table 2. The results have not been corrected for losses except in the case of glutamic acid where the 3 per cent. correction recommended by Moore and Stein (1951) has been applied. No attempt has been

TABLE 2
AMINO ACID COMPOSITION OF HYDROLYSATES OF RUMEN MICROBIAL PREPARATIONS
Amino acid nitrogen expressed as percentage of total nitrogen

Amino Acid	Bacteria				Protozoa			
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 1	Diet 2	Diet 3	Diet 4
Aspartic acid	6.8	6.7	6.8	6.8	7.4	7.8	8.2	8.4
Threonine	3.5	3.6	3.6	3.8	3.1	3.4	3.5	3.7
Serine	2.8	2.5	2.8	3.0	2.6	3.0	3.2	3.1
Glutamic acid	6.6	7.0	7.5	6.9	7.9	8.3	8.7	8.4
Proline	2.8	2.1	2.4	2.2	1.9	2.9	2.1	2.2
Glycine	6.0	6.3	5.9	6.1	4.9	5.7	4.7	5.7
Alanine	6.5	6.5	6.4	6.5	4.1	4.6	4.1	4.1
Cystine	0.7	0.8	0.8	0.8	1.1	1.1	1.3	1.2
Valine	4.4	4.5	4.4	4.5	3.6	4.1	3.7	4.0
Methionine	1.5	1.5	1.5	1.5	1.0	1.4	1.4	1.4
Isoleucine	3.6	3.7	3.6	3.8	4.3	4.6	4.9	4.5
Leucine	4.5	4.6	4.7	4.6	5.0	5.7	5.3	5.2
Tyrosine	2.0	2.1	2.2	2.1	2.0	2.3	2.2	2.4
Phenylalanine	2.3	2.5	2.5	2.4	2.8	3.3	3.2	3.2
Histidine	3.0	2.6	2.6	2.8	2.6	3.4	2.9	3.1
Lysine	8.2	7.5	7.9	8.2	10.7	10.6	11.3	12.6
Arginine	9.1	8.6	9.3	9.3	8.1	10.0	8.4	9.3

made to translate the amino acid compositions of the hydrolysates to those of the original microbial proteins so that as far as the latter are concerned the values, particularly for serine, threonine, methionine, and arginine are probably low. The quantitative determination of tryptophan was not attempted.

III. DISCUSSION

The uniformity of the results for the bacterial hydrolysates indicates that for a wide range of types of diet the amino acid composition of the mixed bacterial proteins is almost constant. This amino acid pattern is similar to that found by Duncan *et al.* (1953) in the rumen digesta of calves fed rations in which the bulk of the dietary nitrogen was supplied by urea. This is shown in Table 3 together with a comparison of the bacterial and protozoal hydrolysates with published values for a range of

herbage proteins. Although the basis for comparing such sets of values may be weakened by the differences in analytical techniques and probable differences in losses during hydrolysis it is evident that the mixture of bulk proteins of rumen bacteria has an amino acid distribution similar to that of the bulk protein of plant leaves. Thus it seems probable that for sheep consuming a normal herbage diet, the conversion of plant to bacterial protein in the rumen would not greatly affect the relative amounts of amino acids in the protein mixture which becomes available for digestion in the abomasum and intestines.

TABLE 3

AMINO ACID COMPOSITION OF RUMEN MICROBIAL HYDROLYSATES COMPARED WITH RUMEN DIGESTA AND HERBAGE PROTEINS

Amino acid nitrogen expressed as percentage of total nitrogen

Amino Acid	Bacteria	Protozoa	Rumen Digesta*	Herbage Proteins
Aspartic acid	6.7-6.8	7.4-8.4	—	4.7-5.4†
Threonine	3.5-3.8	3.1-3.7	3.1-4.6	3.7-4.2
Serine	2.5-3.0	2.6-3.2	—	3.2-3.8
Glutamic acid	6.6-7.5	7.9-8.7	—	6.4-7.8†
Proline	2.1-2.8	1.9-2.9	—	3.5-3.8
Glycine	5.9-6.3	4.7-5.7	—	6.4-7.0
Alanine	6.4-6.5	4.1-4.6	—	5.7-6.0
Cystine	0.7-0.8	1.1-1.3	—	1.1-1.6†
Valine	4.4-4.5	3.6-4.1	3.6-4.5	4.5-4.8
Methionine	1.5	1.0-1.4	0.5-0.9	1.2-1.6†
Isoleucine	3.6-3.8	4.3-4.9	2.2-4.1	3.3-3.5
Leucine	4.5-4.7	5.0-5.7	3.7-5.1	5.3-5.9
Tyrosine	2.0-2.2	2.0-2.4	—	1.5-1.7
Phenylalanine	2.3-2.5	2.8-3.3	1.7-2.1	3.3-3.6
Histidine	2.6-3.0	2.6-3.4	2.7-4.0	3.6-4.0†
Lysine	7.5-8.2	10.6-12.6	4.6-7.9	5.0-6.8†
Arginine	8.6-9.3	8.1-10.6	9.5-12.0	12.0-14.0†

* Calculated from data of Duncan *et al.* (1953).

† Data given by Lugg (1949). The remaining values in this column are from the data of Kemble and Macpherson (1954), with threonine and serine values uncorrected for hydrolysis losses.

The protozoal hydrolysates are of somewhat less uniform composition than the bacterial, possibly because of the smaller number of species represented in the mixture. In feeding trials with rats, McNaught *et al.* (1954) demonstrated a higher nutritive value for protozoal protein compared with bacterial in fractions of bovine rumen microorganisms and this might be explained in terms of amino acid distribution. There is evidence (Black, Kleiber, and Smith 1952) that the amino acids for which dairy cows are dependent on dietary and microbial protein are the ones considered essential for rats. Comparing the "essential" amino acids of the bacterial with the protozoal hydrolysates the more important differences are the higher values

with protozoa for isoleucine, leucine, phenylalanine, and, in particular, lysine. These differences may help to account for the apparent nutritional superiority of the protozoal fraction of rumen microbial proteins.

IV. ACKNOWLEDGMENT

Grateful acknowledgment is made to Mr. V. A. Stephen who designed the fraction collector, reagent dispensers, and other equipment.

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THE DEVELOPMENT OF THE EPIDERMIS AND HAIR CANALS IN THE MERINO SHEEP FOETUS

By A. G. LYNE*

[Manuscript received February 15, 1957]

Summary

A study has been made of the development of the epidermis and hair canals in Merino sheep foetuses ranging in age from 69 days to birth. The epidermis from several Merino lambs and adults has also been examined.

The keratinization of the epidermis, which takes place after the emergence of the first wool fibres, has been described in relation to the development of the wool follicles.

The development of hair canals in primary and secondary wool follicles in Merino sheep has been described for the first time. This is found to be a function both of the sebaceous gland cells which degenerate in the neck of the follicle, and of epidermal cells which keratinize at a separate locus.

I. INTRODUCTION

Although recent workers have devoted much attention to the development of wool follicles in sheep few studies have been made of the epidermis. Similarly, the formation of hair canals in sheep has not been adequately described.

In the present study the differentiation of the epidermis and hair canals is related to the "F" stages of wool follicle development as described by Hardy and Lyne (1956*b*, 1956*c*). Particular attention is given to the epidermis on the fleece-bearing area and no observations on the condition of the epidermis prior to the first appearance of wool follicles are recorded here. The "F" stages of follicle development were described under the following headings: *F1*, follicle plug; *F2*, pre-papilla; *F3*, papilla; *F4*, hair cone; *F5*, advanced hair cone; *F6*, hair formation; *F7*, hair in epidermis; *F8*, hair emerged. For primary (*P*) follicles, stage *F2* was divided into stage *F2a*, when the rudiment of the sudoriferous gland appears, and stage *F2b*, when a sebaceous gland with differentiated cells is first recognized. In both *P* and secondary (*S*) follicles stage *F3* was divided into *F3a*, when the dermal papilla at the base of the follicle has a depth less than its diameter, and *F3b*, when the depth of the papilla is equal to or (rarely) greater than its diameter.

II. MATERIAL AND METHODS

Twenty-four sheep foetuses (ranging in age from 69 to 145 days) described in a recent paper by Hardy and Lyne (1956*c*) provided some of the material for the present study. The other material examined came from eight medium-woolled Merino foetuses of known age (from 75 to 138 days). The epidermis of several Merino lambs and adults was also examined. The sampling positions, all from the fleece-bearing area of the trunk, included a midside sample for every foetus. Additional samples from the coronet region of two foetuses were used in the study of hair canal development.

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The histological methods were similar to those described by Hardy and Lyne (1956c). Fixation was with formalin or Zenker's fluid and sections were cut at 7 or 8 μ , occasionally thicker, and were stained with either haemalum and eosin or haemalum, eosin, and picric acid.

The terminology for wool follicles is that described by Hardy and Lyne (1956a). The terminology for epidermal strata is that defined in Hanson's (1947) detailed study of epidermal histogenesis in the rat and mouse, and is similar to that used by Pinkus (1910) for human material.

III. DEVELOPMENT OF THE EPIDERMIS

From stage *F1* to stage *F7* of the central primary (*PC*) wool follicles, the epidermis on the lateral regions of the trunk consists of three layers: the stratum germinativum, the stratum spinosum, and the periderm (Figs. 1(a) and 1(b)). When the *PC* follicles reach stage *F8* the periderm is replaced by the stratum corneum (Figs. 1(c) and 1(d)). In all the post-natal material examined the epidermis remains thin and retains the same simple structure that it had at birth (Fig. 1(e)).

(a) *Stratum Germinativum*

The stratum germinativum, or basal layer of the epidermis, consists of undifferentiated cells which give rise to the other epidermal strata as well as the first wool follicles (Fig. 1(a)). Its cells usually form a conspicuous single layer adjacent to the basement membrane, and the nuclei, at first cuboidal in shape, are very large in proportion to the volume of the cells. The outlines of the cells themselves are irregular and indistinct.

When the first *S* fibres emerge the stratum germinativum is still a continuous layer but it may be an incomplete layer at birth (Fig. 1(e)).

(b) *Stratum Spinosum*

The stratum spinosum, superficial to the stratum germinativum, is well established when only the *PC* follicles are present and it can be easily recognized as a continuous layer when the first fibres emerge (Fig. 1(c)). It consists of differentiating cells with relatively more cytoplasm and more clearly defined cell outlines than the cells of the stratum germinativum. Three or four cell layers can be recognized and the cells become flatter as they approach the skin surface. Within the stratum spinosum the first indication of the formation of some of the hair canals (described in Section IV) is seen when the *PC* follicles reach stage *F2b*.

When the first *S* fibres emerge, the stratum spinosum still consists of several layers but it is no more than an incomplete single layer of cells at birth (Fig. 1(e)).

(c) *Periderm and Stratum Corneum*

The periderm is the outermost layer of the epidermis and, on the lateral regions of the trunk, it persists at least until the first *PC* follicles reach stage *F8* (Fig. 1(c)). The cells usually have dense eosinophilic cytoplasm. In sections cut at right angles to the skin surface the periderm cells appear to be flattened and have deeply staining

flattened nuclei; when viewed from the surface they appear as large polygonal cells with distinct cell outlines and pale round nuclei. Throughout this period the periderm appears to be a continuous layer one or two cells thick.

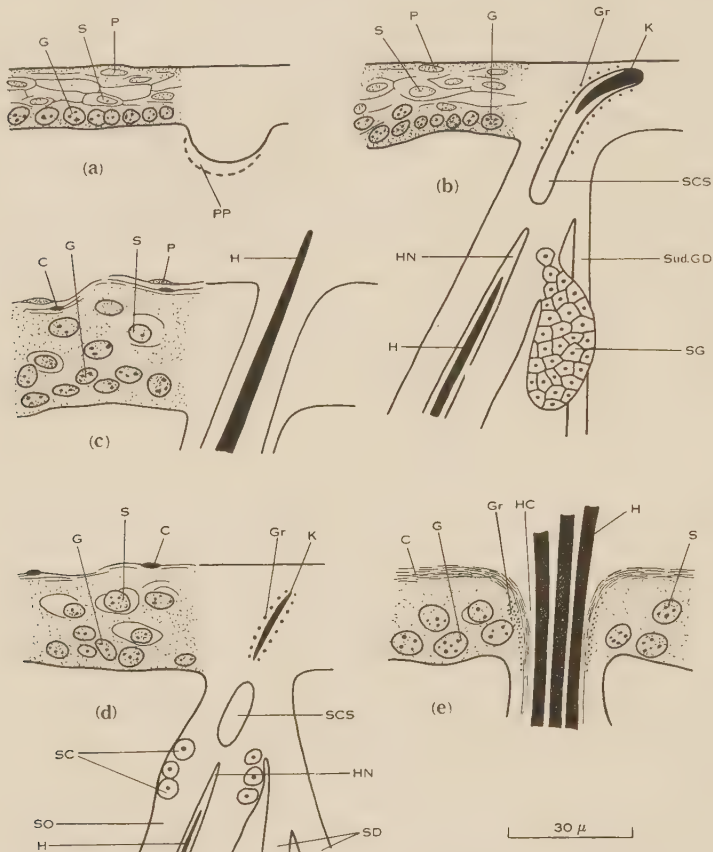


Fig. 1.—Camera lucida drawings of the epidermis of Merino fetuses during the period of wool follicle development. *C*, stratum corneum; *G*, stratum germinativum; *Gr*, cells containing keratohyalin granules; *H*, wool fibre; *HC*, hair canal; *HN*, hair cone; *K*, keratinized cells of hair canal; *P*, periderm; *PP*, pre-papilla; *S*, stratum spinosum; *SC*, sebaceous cells; *SCS*, space formed by disintegration of sebaceous cells; *SD*, derived secondary follicles; *SG*, sebaceous gland; *SO*, original secondary follicle; *Sud.G.D*, sudoriferous gland duct. (a) Lateral lumbar region of 69-day fetus. The most advanced follicles are at state *F1*. (b) Mid-lateral region of trunk of 98-day fetus. Most advanced follicles at stage *F6*. (c) Posterior cervical region of 101-day fetus. Most advanced *PC* follicles at stage *F8*. (d) Sternal region of 108-day fetus. Most advanced *SO* follicles at stage *F6*. (e) Mid-lateral region of trunk 4 days after birth.

When the first *PC* fibres emerge, cells containing small flattened pyknotic nuclei are seen immediately below the periderm (Fig. 1(c)). These nucleated cells constitute the beginning of the stratum corneum which is at first parakeratotic (i.e. it shows imperfect cornification). For a time the periderm cells remain above the stratum corneum but none can be distinguished after the first *S* follicles reach stage *F6* (Fig. 1(d)). The cells of the periderm become greatly flattened but they do not pass through

the same stages of keratinization as are seen in the formation of the definitive stratum corneum.

After the emergence of the *S* fibres, the stratum corneum has its typical structure with several layers of cornified cells, and the pyknotic nuclei have disappeared (Fig. 1(e)).

(d) *Stratum Granulosum*

The stratum granulosum is absent or very poorly developed, except around the follicles and in the neck regions of the follicles (Fig. 1(e)).

IV. DEVELOPMENT OF THE HAIR CANALS

The hair canals in the Merino foetus are formed by two separate processes—keratinization of cells in the epidermis and disintegration of sebaceous cells which have migrated to the neck of the follicle. The initial stages of differentiation of the epidermal cells are independent of those of sebaceous cells and the two processes are described separately. In the epidermal cells there is keratohyalin granulation and keratinization which is followed by the appearance of an intercellular space, while in the sebaceous cells there is cell degeneration followed by space formation. Eventually a continuous canal runs from the follicle neck to the upper part of the epidermis.

(a) *Stages of Hair Canal Development*

Epidermal Stages

Stage A: Granulation.—This stage begins with the appearance of keratohyalin granules within one or more cells of the stratum spinosum, immediately above the ental (obtuse angle) side of the follicle, or within the upper neck region of the follicle.

Stage B: Keratinization.—The granulation extends to the surrounding cells and those in the centre become keratinized (as judged by the appearance of thin sheets of keratin).

Stage C: Space Formation.—In fixed material a tube-shaped space forms within the region of keratinizing cells.

Sebaceous Cell Stages

Stage A: Migration.—Differentiated cells from the sebaceous gland move into the neck region of the follicle as isolated cells or as a column of cells connected with the gland.

Stage B: Degeneration.—The sebaceous cells degenerate (i.e. the nuclei and cell outlines disappear) within the neck region of the follicle.

Stage C: Space Formation.—As the sebaceous cells degenerate one or more spaces filled with fat and detritus are formed within the follicle neck.

Completed Hair Canal

The hair canal extends from above the sebaceous gland to the level of the stratum corneum.

Some or all of the above stages may be recognized in the development of both *P* and *S* follicles (Fig. 2; Plates 1 and 2).

(b) *Primary Follicles*

The epidermal stages in the formation of the hair canal of a *P* follicle usually occur within the stratum spinosum of the epidermis. Epidermal stage A (Fig. 2(a); Plate 1, Fig. 1) usually precedes sebaceous cell stage A but these two stages may begin at about the same time (Fig. 2(b); Plate 1, Fig. 2), when the follicle is at stage *F3a* or *F3b*. In skin from the coronet, epidermal stage A has been observed as early as follicle stage *F2b*. Further development of the epidermal portion of the hair canal proceeds upwards and downwards approximately in the direction of the long axis of the follicle until it reaches the periderm above and the level of the stratum germ-

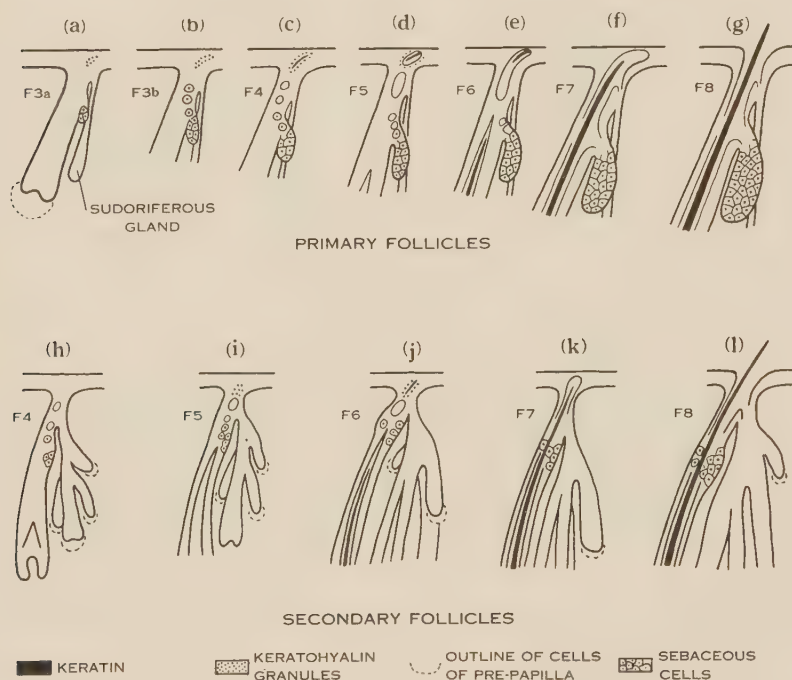


Fig. 2.—Stages in the development of hair canals in the Merino foetus. (a)–(g), primary follicles; (h)–(l), secondary follicles.

inativum below. Epidermal stage B usually appears at the same time as sebaceous cell stage B (Fig. 2(c); Plate 1, Fig. 4) but, in skin from the coronet, it may begin at sebaceous cell stage A (Plate 2, Fig. 1). Sebaceous cell stage C usually precedes epidermal stage C (Fig. 2(d); Plate 1, Fig. 3; Plate 2, Fig. 2).

The hair canal may be completed (Fig. 2(f); Plate 1, Fig. 5) as early as stage *F3b* but this more frequently occurs at stage *F4* to *F5*. At stage *F7* the hair canal is always completed. The upper part of the completed hair canal is usually bent so that it is almost parallel with the skin surface.

No difference between the manner of hair canal formation in central and lateral primary follicles has yet been observed.

(c) *Secondary Follicles*

The formation of the hair canal of an original secondary (*SO*) follicle (Figs. 2(*h*)-2(*l*); Plate 1, Figs. 6-9) is similar in most respects to that described above for a *P* follicle, except that:

- (i) Epidermal stage A (Fig. 2(*i*); Plate 1, Fig. 7) usually occurs later than sebaceous cell stage A (Fig. 2(*h*); Plate 1, Fig. 6) and neither has been observed before follicle stage *F4*.
- (ii) Epidermal stage A frequently begins within the upper neck region of the follicle rather than in the epidermis proper.
- (iii) The *SO* follicle provides the common hair canal for all of the derived secondary (*SD*) follicles (Figs. 2(*h*)-2(*l*); Plate 1, Figs. 6 and 8) which may arise from it.
- (iv) The characteristic bending of the upper part of the hair canal of *P* follicles has not been observed with *S* follicles (Plate 1, Fig. 9).

V. DISCUSSION

In the Merino sheep the first cornified layers of the epidermis were parakeratotic, and were not replaced by a normal stratum corneum until the first *S* fibres had emerged from the skin surface. As the *P* fibres had already begun to emerge before the parakeratotic layers were formed, there was no epitrichium in the sense used by Hanson (1947) of "the keratinized layers which are shed when the first hair coat emerges". Pinkus (1910) and Hanson (1947) considered that differentiation of the periderm probably takes place by parakeratosis in the human and in the rat and mouse.

The fully differentiated epidermis of the fleece-bearing area remained thin up to the time of birth and throughout post-natal life, and lacked a continuous stratum granulosum. This is in agreement with the observations of Spöttel and Tänzer (1923) who considered that the reason for the relatively slight development of the epidermis in sheep must be sought in the dense hairy covering.

The keratinization process in the hair canals was different to that in the epidermis. It began much earlier in foetal life, and the cornified cells differentiated from cells loaded with keratohyalin granules. After the emergence of the wool fibres the follicle necks continued to have a keratohyalin layer (stratum granulosum) beneath the cornified cells.

Diem (1907) briefly described hair canal development in the sheep and his observation that the formation of the hair canal is a function both of the sebaceous gland cells, which degenerate in the neck region of the follicle, and of epidermal cells, which keratinize some distance above them, has been confirmed. Diem considered that the sebaceous glands are only secondarily responsible for the formation of the epidermal portion of the hair canal, in that they possibly widen the canal by means of the secretion introduced into the lumen. Later authors appear to have overlooked the observations made by Diem. Duerden and Ritchie (1924) attributed hair canal formation to the disintegration of cells within the solid follicle neck, near the place of origin of the sebaceous gland; at the same time, similar changes

take place among the sebaceous cells so that a short duct is formed. The observations of Marks (1895), Spöttel and Tänzer (1923), and Wildman (1932) are incomplete in that they attributed the origin of the hair canals to the activity of the sebaceous cells alone.

The epidermal stages in the formation of hair canals in mouse pelage hair follicles (Hardy 1949) and mouse vibrissa follicles (Davidson and Hardy 1952) are similar to those described for wool follicles in sheep, except that the keratinization process for vibrissa follicles begins at the surface of the epidermis. No connection between sebaceous gland cells and hair canal formation in the mouse was observed by these authors.

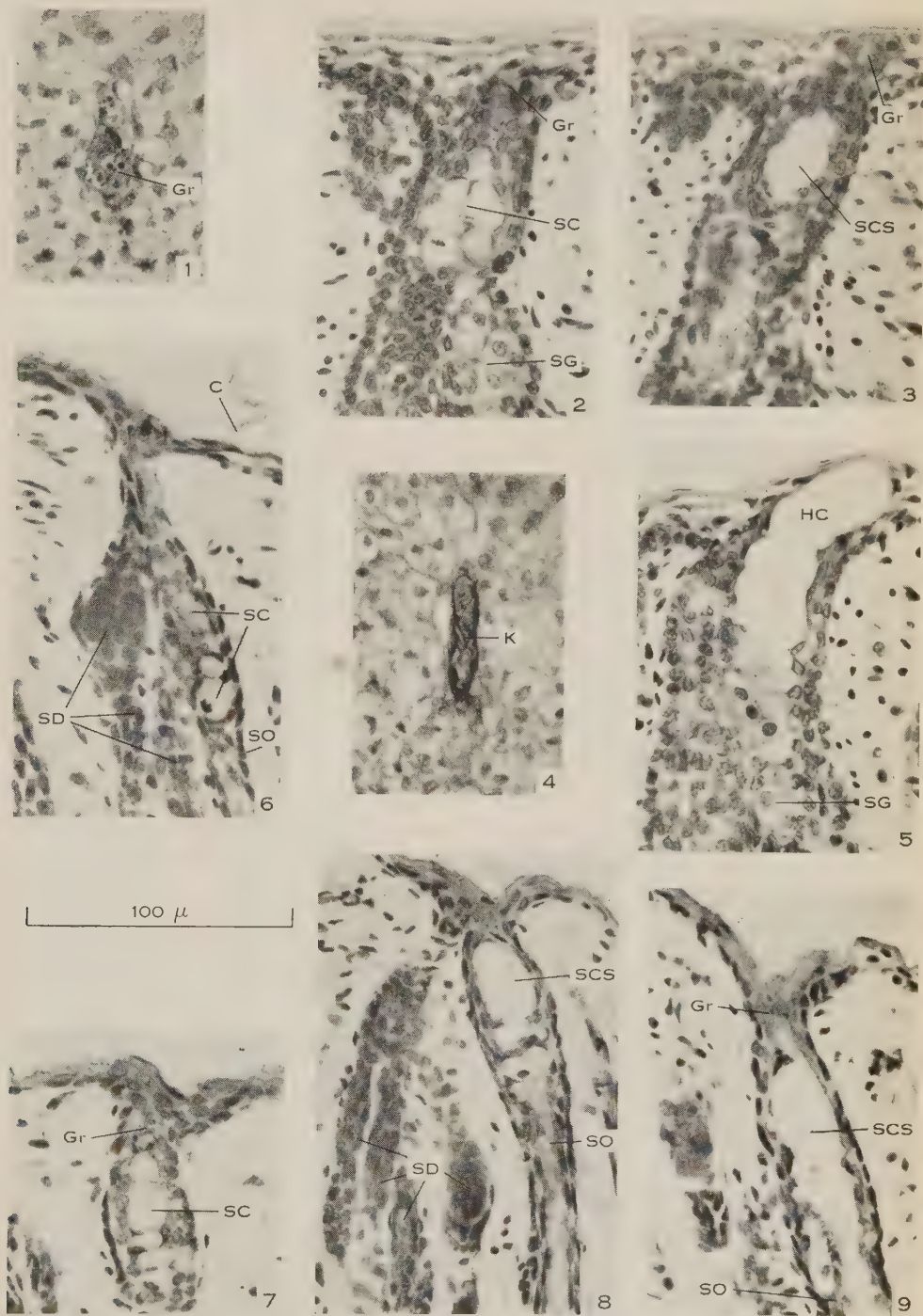
VI. ACKNOWLEDGMENTS

The author is indebted to Professor K. W. Cleland, Department of Histology and Embryology, University of Sydney, for reading the manuscript, and to the following for the loan or supply of material: Mr. H. B. Carter, Animal Breeding Research Organization, Edinburgh; Mr. R. H. Hayman, Officer-in-Charge, F. D. McMaster Field Station, and Dr. B. F. Short, Sheep Biology Laboratory, Prospect, both of the Division of Animal Health and Production, C.S.I.R.O. The assistance of Miss M. J. Heideman in the preparation of sections, and of Mr. I. T. Roper in photography is gratefully acknowledged.

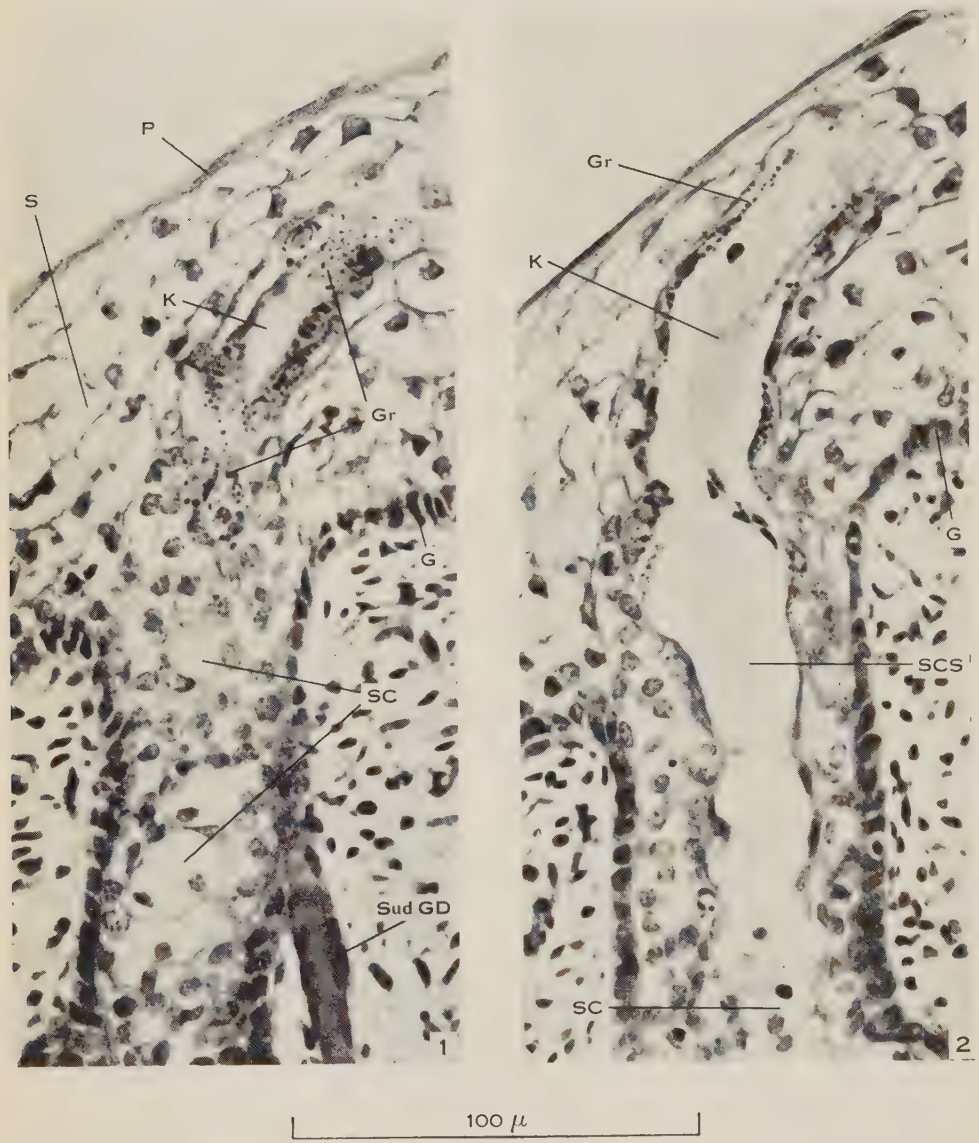
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EPIDERMIS AND HAIR CANALS IN MERINO SHEEP



EPIDERMIS AND HAIR CANALS IN MERINO SHEEP



EXPLANATION OF PLATES 1 AND 2

All figures are of sections through skin from Merino foetuses. The staining is with haemalum, eosin, and picric acid. Lettering as for Figure 1

PLATE 1

Development of hair canals. Figs. 1-5.—Primary follicles: sections from mid-lateral region of the trunk. Figs. 6-9.—Secondary follicles: longitudinal sections of upper parts of *SO* follicles from shoulder region of 125-day foetus

Fig. 1.—Horizontal section of epidermis from 81-day foetus with hair canal of *P* follicle at epidermal stage A.

Fig. 2.—Vertical section of upper part of skin from 98-day foetus with hair canal of *P* follicle at epidermal stage A and sebaceous cell stage A.

Fig. 3.—Vertical section of upper part of skin from 98-day foetus with hair canal of *P* follicle at epidermal stage A and sebaceous cell stage C.

Fig. 4.—Horizontal section of epidermis from 91-day foetus with hair canal of *P* follicle at epidermal stage B.

Fig. 5.—Vertical section of upper part of skin from 98-day foetus with completed hair canal of *P* follicle.

Fig. 6.—Hair canal of *SO* follicle at sebaceous cell stage A.

Fig. 7.—Hair canal of *SO* follicle at epidermal stage A. and sebaceous cell stage B.

Fig. 8.—Hair canal of *SO* follicle at sebaceous cell stage C.

Fig. 9.—Hair canal of *SO* follicle at epidermal stage A and sebaceous cell stage C.

PLATE 2

Development of hair canals. Vertical sections of upper part of skin from coronet region of 80-day foetus (believed to be Merino)

Fig. 1.—Hair canal of *P* follicle at epidermal stage B and sebaceous cell stage A. Note that the hair canal is formed by two separate processes—keratinization of cells in the epidermis and disintegration of sebaceous cells which migrate to the neck of the follicle.

Fig. 2.—Hair canal of *P* follicle at early epidermal stage C and sebaceous cell stage C. Note the keratohyalin layer beneath the cornified cells within the epidermal portion of the hair canal.

NUCLEIC ACIDS AND THE BIOSYNTHESIS OF COLLAGEN

By P. H. SPRINGELL*

[Manuscript received February 6, 1957]

Summary

The study of skin protein fractions obtained by salt fractionation indicates that collagen is associated to a greater extent with the ribonucleoprotein fraction than with the deoxyribonucleoprotein fraction. The gelatin derived from the ribonucleoprotein fraction following *in vitro* incubation of foetal lambskin in the presence of glycine-2-¹⁴C was appreciably radioactive. Pre-treatment of the skin with crystalline ribonuclease resulted in a marked inhibition of glycine incorporation into total gelatin, whereas crystalline deoxyribonuclease had little effect. It is therefore concluded that ribonucleic acid rather than deoxyribonucleic acid is associated with the biosynthesis of collagen.

Methods of extracting collagen from tissues were compared and discussed in relation to possible nucleic acid contamination.

I. INTRODUCTION

The importance of nucleic acids in the biosynthesis of proteins has become increasingly evident in recent years (Brachet 1955). The extension of these studies to collagen has been hampered by its relative metabolic inertness (Orekhovich 1952; Harkness *et al.* 1954), particularly in adult mammals. Although previously *in vitro* studies were confined to long-term experiments (Gerarde and Jones 1953), in the present work a short-term *in vitro* system with foetal sheepskin pieces was made possible by use of the isotopic tracer technique.

II. EXPERIMENTAL

(a) Materials

(i) *Foetal Sheepskin*.—Foetuses (15–23 cm from base of neck to base of tail) from freshly killed ewes were kept warm until ready for incubation within an hour of slaughter. Portions of skin were dissected from the mid-dorsal region.

(ii) *Adult Sheepskin*.—Snippets of skin were taken from the edge of fresh pelts and freed from the bulk of the wool.

(iii) *Kangaroo Tail Tendon*.—This was air-dried material which had not been subjected to any chemical treatment.

(iv) *Commercial Gelatins*.—Davis and Difco gelatins were used.

(v) *Crystalline Enzymes*.—Crystalline ribonuclease (Armour, lot 3014X) and crystalline deoxyribonuclease (Worthington, lot 289) were used.

(vi) *Glycine-2-¹⁴C*.—A 1 mc (19.4 mg) sample of methylene-labelled glycine (Radiochemical Centre, Amersham) was dissolved in 100 ml distilled water, and stored in the frozen state until required.

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(b) *Methods*

(i) *Incubation*.—About 2 g (wet weight) foetal skin was placed in an incubating medium consisting of 40 ml of solution with a final concentration of 0.13M potassium phosphate buffer, pH 7.4, 0.13M sodium chloride, 1:20,000 "Merthiolate", and 2 ml radioglycine solution. In experiments where pre-incubation with nucleases was undertaken, the medium consisted in the first instance of 20 ml 0.05M potassium phosphate buffer, pH 7.4, containing 0.003M magnesium sulphate and 2 ml radioglycine solution with or without 5 mg of the appropriate nuclease. At the termination of the pre-incubation period of 2 hr, a further 20 ml of solution containing 0.25M potassium phosphate buffer, pH 7.4, 0.26M sodium chloride, and 1:10,000 "Merthiolate" was added. The incubation and pre-incubation were carried out in 150 ml bubblers (5 by 15 cm) for a total of between 28 and 32 hr at 37°C in a slow stream of moist oxygen containing 5 per cent. carbon dioxide. Four incubations were carried out simultaneously. Controls were treated similarly, but were kept at 0°C without gassing.

(ii) *Isolation of Skin Proteins*.—The skin pieces were removed from the medium, blotted on filter paper, and stored in 50-ml centrifuge tubes at -15°C before further treatment. Fresh foetal or adult sheepskin in non-radioactive experiments were also frozen before use.

(1) *Extraction of Skin by Trichloroacetic Acid*.—The procedure used was a modification of the method of Fitch, Harkness, and Harkness (1955). Skin pieces were extracted with 5 ml of 10 per cent. trichloroacetic acid for 15 min at 90°C. The supernatant was collected by centrifugation, the precipitate disintegrated with 10 ml of 10 per cent. trichloroacetic acid in a glass homogenizer, and the heat treatment and centrifuging were then repeated. The residue was then washed twice with 5 ml of 10 per cent. trichloroacetic acid and the pooled extracts were clarified by centrifugation and filtration. The supernatants were dialysed against several changes of distilled water for at least 72 hr. In experiments with labelled glycine, about 0.5 g of non-radioactive glycine was added prior to dialysis. The dialysed protein was again filtered, and an aliquot withdrawn for examination in ultraviolet light and dry weight determination if necessary. In radioglycine experiments the bulk was precipitated by mixing seven volumes of gelatin solution with one volume of 1M sodium chloride and pouring this into 36 volumes of acetone. The gelatin was allowed to precipitate out overnight at 0°C, and was then collected by centrifugation and finally dried with acetone and ether to yield a fine powder suitable for plating.

The residues were suspended in 5 ml of 10 per cent. non-radioactive glycine overnight (in non-radioactive experiments this was omitted), washed in water, and dried with acetone and ether.

(2) *Extraction of Skin by Autoclaving*.—Skin pieces were autoclaved in 5 ml of distilled water for 3 hr at 15 lb/in.². The extract was collected by centrifugation, and the residue washed twice more with hot water. The combined extracts and washings were filtered and, in radioglycine experiments, 0.5 g of non-radioactive glycine was added before dialysis. The dialysed protein and the insoluble material remaining after autoclaving were worked up as in the trichloroacetic acid procedure.

(3) *Fractionation of Skin Proteins*.—The procedure used was based on that of Mirsky and Pollister (1946) and Wiest and Heidelberger (1953). The thawed skin pieces were disintegrated with 6 ml of 0.14M sodium chloride – 0.01M potassium phosphate buffer, pH 6.8, in a glass homogenizer. The residue was collected by centrifugation and was then re-extracted twice more with sodium chloride–potassium phosphate. The pooled supernatants were clarified by further centrifugation, approximately 0.5 g of non-radioactive glycine was added, followed by 2 ml of 5M acetic acid to precipitate the ribonucleoprotein which was then allowed to settle overnight. The ribonucleoprotein was washed with 0.14M sodium chloride, distilled water, and then dried with acetone and ether before plating.

The residue was extracted with 6 ml of 1M sodium chloride and left overnight in the cold before removal of the supernatant by centrifugation. The precipitate was re-extracted with two further lots of 1M sodium chloride and the combined super-

TABLE 1
RECOVERY OF EXTRACTS AND RESIDUES FROM SHEEPSKIN

	Foetal	Adult
Wet weight of whole skin (mg)	2000	2000
Dry weight of whole skin (mg)	224	510
Extraction with trichloroacetic acid		
Dry weight of dialysed extract (mg)	22	127
Dry weight of residue (mg)	127	252
Extraction by autoclaving		
Dry weight of dialysed extract (mg)	73	187
Dry weight of residue (mg)	83	183

natants were clarified by further centrifugation and filtration. After the addition of 0.5 g of non-radioactive glycine, the deoxyribonucleoprotein fraction was dialysed against 1M sodium chloride for 72 hr, then precipitated with 5 per cent. trichloroacetic acid, washed with more trichloroacetic acid, and then dried with acetone and ether before plating.

Subsequent to counting, both nucleoprotein fractions were autoclaved and gelatins isolated by the procedure described above.

(iii) *Treatment of Commercial Gelatins and Kangaroo Tail Tendon*.—The gelatins were either dissolved and examined as such, or dialysed as well; in other instances they were subjected to the autoclaving or trichloroacetic acid treatments. Kangaroo tail tendon collagen was converted to gelatin by means of trichloroacetic acid or by autoclaving.

(iv) *Ultraviolet Spectrophotometry*.—A Beckman model DU spectrophotometer was used. The samples examined were diluted to yield solutions of 0.01–0.02 per cent. The $E_{1\text{cm}}^{1\%}$ values were calculated following dry weight determinations on suitable aliquots.

(v) *Radioactivity Measurements*.—Aluminium discs (25 mm diameter, 4 mm thick, 1 cm² well) were used for mounting radioactive samples. As far as possible sufficient material was used to give samples of infinite thickness, but where this was not possible, counts were corrected to infinite thickness. A ± 5 per cent. standard error of counting was not exceeded. Radioactivities were measured using a thin-end window Geiger-Müller tube (EHM 2^s) with a conventional scaler unit.

III. RESULTS

While investigating the most suitable method for extraction of total collagen from sheepskin, it was observed that when both adult and foetal skins were extracted

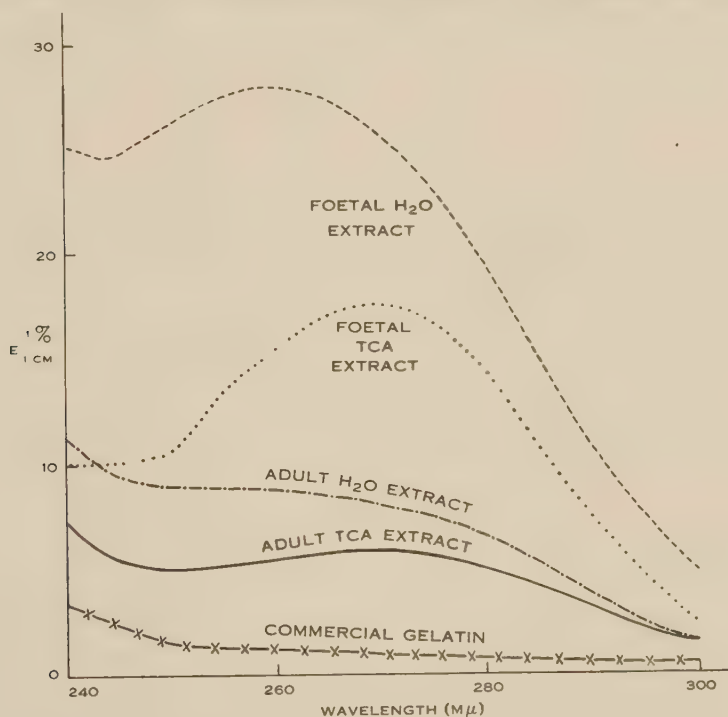


Fig. 1.—Ultraviolet absorption spectra of extracted adult and foetal sheepskin (from foetus No. 21) and of commercial gelatin (undialysed Davis gelatin). TCA, trichloroacetic acid.

with hot trichloroacetic acid, less material was removed than by autoclaving (Table 1). Furthermore, the dry weights of the dialysed trichloroacetic acid extracts were smaller than those obtained by the alternative procedure.

The extracts obtained by the two methods also differed in their ultraviolet absorption characteristics (Fig. 1). The absorption peaks of the trichloroacetic acid extracts were located at 270 mμ, while those of the autoclaved extract were at 260 mμ. The extinction coefficients of the latter were also higher. For adult sheepskin extracts the absorption curves were lower and much flatter in the 250–280 mμ region.

Commercial gelatins showed a much lower absorption than was found with skin extracts. Moreover, neither autoclaving nor trichloroacetic acid treatment had any influence on its absorption characteristics. As with skin, kangaroo tail tendon gave a larger yield of gelatin when extracted by autoclaving than by the alternative method, but the ultraviolet absorption characteristics of the two extracts were similar and closely resembled those of commercial gelatin.

TABLE 2
RADIOACTIVITY OF GELATIN EXTRACTED FROM FOETAL SHEEPSKIN
FOLLOWING IN VITRO INCORPORATION OF RADIOGLYCINE

Extraction Procedure	Foetus 19 (counts/min)	Foetus 20 (counts/min)	Foetus 23 (counts/min)
Trichloroacetic acid	20	68	2544
Autoclaving	131	168	8180

When foetal sheepskin was incubated with radioglycine and the radioactivity of the total gelatins isolated by the two procedures compared, it was evident (Table 2) that more radioactivity had been incorporated into gelatin obtained by autoclaving.

TABLE 3
DISTRIBUTION OF RADIOACTIVITY IN TOTAL AND FRACTIONATED FOETAL SHEEPSKIN

Fraction	Foetus 14 (counts/min)		Foetus 15 (counts/min)	
	Incubated	Control	Incubated	Control
Total skin gelatin	1490	58	1660	63
Residual skin	658	32	1000	40
Total ribonucleoprotein	1902	70	372	72
Ribonucleogelatin	2424	23	263	31
Ribonucleoprotein residue	1220	14	165	14
Total deoxyribonucleoprotein	282	10	94	10
Deoxyribonucleoprotein residue	1165	19	530	25

In subsequent work, the autoclaving rather than the trichloroacetic acid technique was adopted, because of the better yield of gelatin and the higher specific activity of the gelatin obtained in this way.

The distribution of radioactivities in two fractionation experiments is given in Table 3. It was found that in each case more glycine was incorporated into the incubated samples than into the controls, and that the soluble protein* derived from

*Although no attempt at quantitative recovery was made, this material amounted to about 10 mg.

the autoclaved ribonucleoprotein fraction had incorporated appreciable radioactivity. Although direct radioactivity measurements of the gelatin derived from the deoxyribonucleoprotein fraction could not be carried out because only traces of this gelatin were recovered, the indications were that since the deoxyribonucleoprotein residue after autoclaving showed a sharp rise in radioactivity, only relatively inert or weakly radioactive material had been removed by the autoclaving.

TABLE 4
INFLUENCE OF CRYSTALLINE RIBONUCLEASE AND DEOXYRIBONUCLEASE ON GLYCINE INCORPORATION INTO TOTAL SKIN GELATIN

Nature of Pretreatment	Foetus 16 (counts/min)	Foetus 17 (counts/min)	Foetus 22 (counts/min)	Foetus 23 (counts/min)
None (cold control)	—	—	51	67
None (incubated)	1534	1870	1036	8180
Ribonuclease	645	—	536	3919
Deoxyribonuclease	—	1751	1318	8470

Pre-incubation of sheepskin with both ribonuclease and deoxyribonuclease was undertaken and the effect on glycine incorporation noted. Table 4 shows that ribonuclease inhibits glycine uptake into the total skin gelatin fraction by a factor of two, whereas deoxyribonuclease has little effect.

Examination of these gelatins in the ultraviolet indicated that both the shape of the absorption curves and the extinction coefficients at the absorption maximum of 260 $m\mu$ (Table 5) remained virtually unaltered as a result of the nuclease action.

TABLE 5
INFLUENCE OF CRYSTALLINE RIBONUCLEASE AND DEOXYRIBONUCLEASE ON $E_{1\text{cm}}^{1\%}$ VALUES OF EXTRACTED GELATINS AT 260 $m\mu$

Nature of Pretreatment	Foetus 16	Foetus 17	Foetus 22	Foetus 23
None (cold control)	—	—	24.3	29.3
None (incubated)	18.7	14.0	23.7	30.5
Ribonuclease	18.3	—	24.0	34.0
Deoxyribonuclease	—	15.0	23.1	30.5

IV. DISCUSSION

The results in Tables 3 and 4 indicate that no glycine incorporation occurs in the absence of oxygen and at low temperature. The fact that collagen was associated with the ribonucleoprotein fraction, that the derived gelatin was labelled to an appreciable extent, and that ribonuclease inhibited glycine incorporation into the total skin gelatin, may mean that ribonucleic acid is associated with collagen synthesis. Conversely, the failure to obtain measurable quantities of gelatin from the deoxyribonucleoprotein fraction, together with the likelihood of the virtual absence of labelling in such gelatin and the absence of an effect by deoxyribonuclease on glycine uptake by total skin gelatin, may be taken as an indication that deoxyribonucleic acid is not concerned with collagen synthesis to an appreciable extent.

The relatively high absorption in the 260 $m\mu$ region of the skin-derived gelatins (Fig. 1) indicates possible contamination with nucleic acids or their degradation products, particularly in the extracts of autoclaved foetal skins. Furthermore, the above findings suggest that the richer the tissue in nucleoprotein, the greater the difference between extracts obtained by trichloroacetic acid treatment and by autoclaving.

Rough estimates of the possible nucleic acid content may be obtained from phosphorus analyses and extinction coefficients by assuming that nucleic acid

TABLE 6
NUCLEIC ACID CONTENT OF GELATINS AS CALCULATED FROM PHOSPHORUS ANALYSES AND
EXTINCTION DATA

Sample	Phosphorus Content (g/100 g gelatin)	$E_{1\text{cm}}^{1\%}$ at 260 $m\mu$	Calculated Nucleic Acid Content (g/100 g gelatin)	
			From Phosphorus Content	From $E_{1\text{cm}}^{1\%}$
Difco gelatin	0.04	1.57	0.4	0.5
Davis gelatin	0.03	1.26	0.3	0.4
Foetus 19				
Autoclaved extract	0.20	20.9	2.2	7.2
Trichloroacetic acid extract	0.15	10.8	1.7	3.7
Foetus 23				
Autoclaved extract	0.10	30.5	1.1	10.5
Trichloroacetic acid extract	0.10	24.0	1.1	8.3

contains 9 per cent. phosphorus and that the extinction per mole of phosphorus is 10,000 (Table 6). It is evident that for commercial gelatins all the nucleic acid could be accounted for in terms of phosphorus, while the extracted gelatins contain less phosphorus than might be expected from the extinction data. One reason for this could be a partial hydrolysis of the phosphate groups during the extraction procedure and their removal by dialysis. Whatever the true explanation, it would appear that the extracted foetal skin gelatins could contain at least 1 or 2 per cent. nucleic acid and possibly even as much as 10 per cent.

The presence of such nucleic acid material could influence the interpretation of results obtained in studies where labelled glycine is used for following collagen metabolism. Because of the known rapid turnover rates of nucleic acids in biological systems (Smellie 1955), incorporation of glycine into nucleic acids would be expected. In fact, Table 2 shows that, following *in vitro* incubation of foetal sheepskin with radioglycine, greater incorporation is associated with the gelatin from the autoclaved extracts, i.e. with the gelatin containing a larger amount of material absorbing at 260 $m\mu$, than with gelatin from the trichloroacetic acid extracts. The practice of isolating the glycine from an acid hydrolysate and taking its radioactivity as a

measure of collagen metabolism may not entirely eliminate the effect due to nucleic acid contamination, because of the possibility of conversion of some of the labelled purines into glycine under conditions of the hydrolysis (Bendich 1955).

If no hydrolysis of the labelled protein is undertaken, as was the case in the present study, and if incubation with nucleases (Table 4) had some influence on the nucleic acid content of the isolated gelatins, then the differences in radioactivity might be a reflection of such changes in nucleic acid content. It would appear from Table 5, however, that the action of nucleases has had no effect on the amount of extractable material absorbing at 260 $m\mu$.

The choice of a suitable extraction method was clearly not an easy one. Similar contamination could be expected in several of the other methods currently used for collagen extraction, particularly from tissues rich in nucleoprotein. Several of these same methods, in fact, have also been employed for extraction of nucleic acids or their derivatives. Thus trichloroacetic acid itself has been used for this purpose (Schneider 1945), while dilute citrate has been used for both procollagen extraction (Orekhovich *et al.* 1948) and for removal of ribonucleic acid from deoxyribonucleic acid (Dounce 1943), and sodium chloride was used for the isolation of salt-soluble collagen (Gross, Highberger, and Schmitt 1955) and of nucleoproteins (Mirsky and Pollister 1946). Although sodium phosphate does not appear to have been used for nucleic acid extraction, Schmitt, Gross, and Highberger (1953) have reported that collagen extracts prepared in this way have strong absorption at 260 $m\mu$.

V. ACKNOWLEDGMENTS

My thanks are due to Mr. F. R. Jackson, Senior Commonwealth Meat Inspector, and Mr. D. A. C. McNicol, Commonwealth Veterinary Officer, at the Footscray factory of Messrs. W. Angliss & Co. Pty. Ltd. for their kind cooperation in supplying the foetal material and sheepskin pieces. Phosphorus analyses were carried out by the Microanalytical Laboratory, C.S.I.R.O., and I wish to thank Dr. K. W. Zimmermann for facilitating these.

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PHYSIOLOGY OF PEA FRUITS

IV. CHANGES IN SUGARS IN THE DEVELOPING SEED

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[*Manuscript received June 14, 1957*]

Summary

Paper chromatographic methods were developed for the estimation of sugars in plant tissues and were used to follow changes in some individual sugars during the development of the pea seed. Sucrose was the predominant sugar present in the seeds and there were smaller quantities of fructose, glucose, and galactose. The amounts of fructose and glucose were approximately equal at all stages of development. The sucrose, fructose, and glucose contents of the seed increased in the early stages of development but decreased during the phase of rapid starch synthesis. The galactose content increased to a maximum at a later state than the other sugars estimated. The concentrations of sucrose, fructose, and glucose in the seeds were compared with the concentrations in the leaves, stems, and hulls. The possible significance of the changes in sugars in relation to the metabolism of the pea seed is discussed.

I. INTRODUCTION

In the first paper of this series, McKee, Robertson, and Lee (1955) showed that the soluble carbohydrate content of pea seeds increased during the early stages of development and subsequently decreased. This decrease coincided with a period of rapid starch formation. The changes in starch and the possible controlling mechanisms of starch synthesis were discussed in a later paper (Turner and Turner 1957). Information on the behaviour of individual sugars in the developing pea is needed to understand the starch-sugar transformations.

As a preliminary to the study of sugar changes, methods for the precise determination of the sugars were examined. A difficulty often encountered in the analysis of plant tissues is the occurrence of relatively large amounts of one or more sugars together with very small amounts of other sugars. In the methods described in this paper, the sugars were separated on paper chromatograms, extracted, and determined by appropriate micromethods. The methods may be suitable for the estimation of sugars in a variety of plant tissues, and have been used also in this Laboratory for the determination of sugars in enzyme reaction mixtures.

The present communication is concerned with the changes in sucrose, fructose, glucose, and galactose during the development of pea seeds. The analyses were carried out on replicate samples of the peas used for studies on starch and starch phosphorylase (Turner and Turner 1957) and phosphate compounds (Rowan and Turner 1957).

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II. MATERIALS AND METHODS

(a) *Sampling of Peas*

The sampling of the peas (*Pisum sativum* L., var. Canner's Perfection) from crops grown in 1954 and 1955 was described previously (Turner and Turner 1957). Twelve samples of pods were taken at intervals of 2-4 days, the first at 12 days from flowering and the last at 40 days from flowering.

(b) *Estimation of Starch*

The starch figures are those reported by Turner and Turner (1957).

(c) *Estimation of Sugars*

(i) *Preparation of Extracts from Tissues*.—To a 10-g portion of the mixed sample of peas 20 ml absolute ethanol was added and the mixture stored at -18°C . There was no detectable hydrolysis of sucrose or changes in the other sugars over a period of 4 months in storage. When required for analysis the mixture was finely ground, centrifuged, and the clear supernatant used for chromatography of the sugars. Extracts from leaves, stems, and hulls were prepared similarly. It was unnecessary to de-ionize the extracts before chromatography.

(ii) *Purification of Filter Paper*.—When filter paper as purchased was used directly for quantitative chromatography, relatively high and variable paper blanks were obtained. As a routine procedure the papers were washed as chromatograms with water for 7 days; this gave a low and constant paper blank. Washing with 2N acetic acid did not further improve the blank values. The papers were finally dried in a current of air at 25°C .

(iii) *Chromatography of Sugars*.—Descending chromatography in an all-glass apparatus maintained at 25°C was employed (Hanes and Isherwood 1949). Whatman No. 3MM paper was used for all quantitative work.

The ethanolic extracts of the plant tissues were applied to the papers from a 12.0- μl micropipette. As many as 12 successive applications were made on each spot, a current of warm air being passed over the paper to ensure rapid removal of the solvent. These multiple applications were necessary when one of the sugars to be determined was present in small amount.

To separate the sugars studied (sucrose, fructose, glucose, and galactose) the ethyl acetate-pyridine-water (80 : 20 : 10 v/v) solvent of White and Secor (1953) was used. The chromatograms were dried at room temperature.

(iv) *Extraction of the Sugars from Chromatograms*.—The developed chromatogram was cut into strips corresponding to the points of application of the extracts. Marker strips were sprayed with the aniline phosphate reagent of Ash and Reynolds (1954) to indicate the positions of the various sugars.

The area of paper containing the sugar to be determined was folded twice, placed in a test tube, and 3 ml of water added. Extraction was continued for 2 hr, the tube being very gently shaken several times in this period; vigorous shaking separated paper fibres and increased the paper blanks. It was found that the sugars were usually completely extracted within 30 min. Appropriate areas of paper were taken for

blank values. Procedures such as refluxing the paper with the extracting solvent were unnecessary.

(v) *Determination of the Sugars*.—Several published methods for the micro-determination of sugars were examined.

The modification by Somogyi (1945) of the method of Nelson (1944) gave variable reagent blanks and sugar calibrations. Various methods based on the use of anthrone (e.g. Johanson 1954) gave high reagent and paper blanks, the latter probably due to the presence of small amounts of filter paper fibres. The calibrations using anthrone methods were not consistent. The resorcinol method of Cole, Hanes, Jackson, and Loughman (see Bell 1955) gave a high and somewhat variable reagent blank and did not give the required sensitivity.

The method of Wager (1954) was found to be very sensitive and gave low paper and reagent blanks and constant calibrations. The following modification was employed for the determination of glucose and galactose. The sample of the sugar solution was diluted to 5 ml with water and 1 ml of copper reagent added. The extinction coefficient was measured without dilution a few minutes after the addition of the arsenomolybdate reagent; the colour was stable for at least 30 min. In the estimation of galactose the time of heating was increased from 10 min (as recommended by Wager) to 20 min. For the determination of sucrose and fructose the slightly more convenient resorcinol method of Roe, Epstein, and Goldstein (1949) was used. This method was of adequate sensitivity and gave low paper and reagent blanks and reproducible calibrations. The sensitivity of the method was increased by measuring the extinction coefficients at 400 $m\mu$ instead of 515 $m\mu$ as recommended by Roe *et al.*

The minimal amounts of sugars which were satisfactorily determined in each spot were 5 μg for glucose, galactose, and fructose and 10 μg for sucrose. Individual estimations of sugars that were applied to papers, developed, and extracted gave recoveries within 4–5 per cent. of the theoretical.

(vi) *The Identification of Galactose*.—As there are few reports of the occurrence of free galactose in plant tissues, positive identification of this sugar was established. The substance thought to be galactose was isolated by applying ethanolic extracts of pea seeds as streaks to Whatman No. 3MM papers. These were developed in pyridine–ethyl acetate–water (80 : 20 : 10 v/v), the unknown sugar extracted, and the solution evaporated to small volume under reduced pressure.

The substance travelled the same distance as an authentic sample of galactose when applied to papers and developed in the following solvents: ethyl acetate–*n*-propanol–water (57 : 32 : 13 v/v) (Wager, unpublished data); ethyl acetate–pyridine–water (20 : 10 : 20 v/v) (Jermyn and Isherwood 1949); ethyl acetate–pyridine–water (80 : 20 : 10 v/v) (White and Secor 1953); phenol–water (Partridge 1948). The last three solvents clearly separate galactose from glucose.

The substance reacted as an aldose when sprayed with benzidine (Bacon and Edelman 1951), naphthoresorcinol (Bryson and Mitchell 1951), aniline phosphate (Ash and Reynolds 1954), and aniline hydrogen phthalate (Partridge 1949).

The osazone of the substance was prepared and recrystallized several times from 40 per cent. ethanol: m.p. 195°C; m.p. galactosazone 196°C.

III. RESULTS

(a) *Concentration of Sugars in Pea Seeds*

The relative concentrations of the several sugars and the nature of the changes involved were very similar in the two seasons. A larger number of samples was taken in 1955 and the results of this season only are presented. Changes in fresh weight, dry weight, and water content per seed have been described in an earlier publication (Turner and Turner 1957). The fresh weight and water content per seed increased until 27 days from flowering and subsequently declined. Dry weight per seed increased until 33 days.

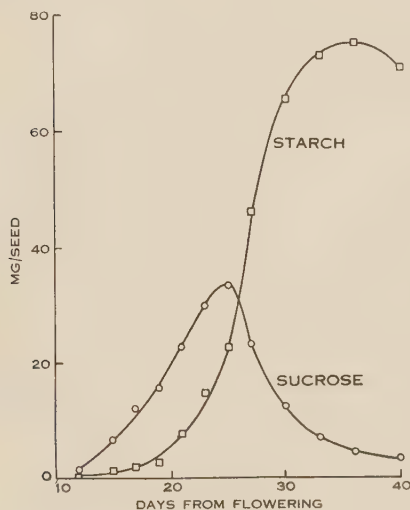


Fig. 1

Fig. 1.—Changes in sucrose and starch in pea seeds with time from flowering.

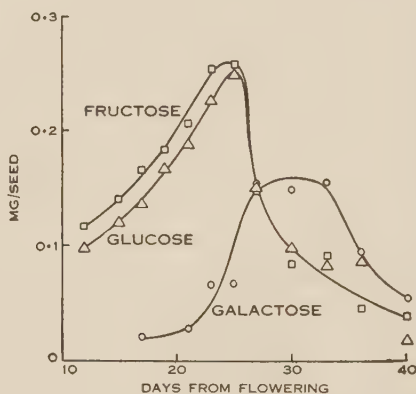


Fig. 2

Fig. 2.—Changes in glucose, fructose, and galactose in pea seeds with time from flowering.

The sucrose content per seed increased steadily from a low value at 12 days from flowering to a maximum at about 25 days and then decreased (Fig. 1). The fructose and glucose contents per seed increased until 25 days from flowering and then decreased sharply (Fig. 2). At the time of the first sampling, both fructose and glucose (in contrast to sucrose) were relatively high, being approximately 40 per cent. of their final maxima. The changes in fructose and glucose were very similar. Galactose changed in a manner different from the other sugars determined. Initially (at 17 days from flowering) the content per seed was very low in comparison with other sugars (Fig. 2). After about 21 days from flowering, the galactose content increased rapidly until 27 days and decreased after 33 days. The changes in starch content per seed are shown in Figure 1. After 21 days from flowering, a phase of rapid synthesis commenced and continued until 30 days.

(b) *Sugars in other Tissues of the Pea Plant*

The concentrations of sugars in other parts of the pea plant were compared with those found in the seeds. Table 1 shows results of analyses of leaves and stems

taken from the experimental plants at 7 a.m. at 30 days from flowering. The hulls analysed, of about the same age, came from another plot.

The sucrose concentration in the leaves was considerably lower than that found in the seeds of any age. The concentrations of fructose and glucose in the leaves were alike (as in the seeds) but were much higher relative to sucrose than in seeds.

The stems and hulls showed a different pattern. In the stems the sucrose and fructose concentrations were low whereas that of glucose was high. In the hulls the concentrations of glucose and sucrose were high and that of fructose relatively low.

TABLE 1
CONCENTRATIONS OF SUGARS IN SOME TISSUES OF THE PEA PLANT

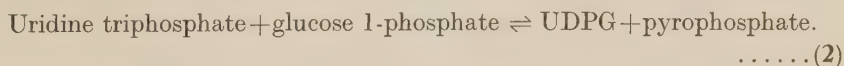
Tissue	Days from Flowering	Sugars (mg/g fresh wt.)		
		Sucrose	Fructose	Glucose
Seeds	12	36.6	3.43	2.87
Seeds	25	66.5	0.51	0.49
Seeds	30	20.8	0.14	0.16
Leaves	30	4.63	2.49	2.34
Stems	30	2.23	0.51	11.6
Hulls	Approx. 25	9.61	1.11	10.5

IV. DISCUSSION

It is believed that the substrates for carbohydrate synthesis transported to the pea are sugars rather than hexose phosphates (Turner and Turner 1957). The sucrose content per seed increased from the first sampling until 25 days from flowering. Starch was then being formed rapidly and although sugars were probably being imported at approximately the same rate, the sucrose content of the seeds decreased. Glucose 1-phosphate is the common precursor both for starch synthesis (Hanes 1940) and sucrose synthesis. Experiments with pea extracts (Turner 1953, 1954; Cardini, Leloir, and Chiriboga 1955) have indicated that sucrose is formed from uridine diphosphoglucose (UDPG) and fructose by reaction (1):



UDPG may be formed by reaction (2):



The enzyme catalyzing reaction (2) has been found in pea extracts in this Laboratory (Turner and Turner, unpublished data).

The phase of rapid starch synthesis commenced about 21 days from flowering and it is probable that this resulted in a decrease in the available concentration of glucose 1-phosphate. Rowan and Turner (1957) using replicate samples of the pea seeds taken for the present experiment, observed a decrease after 23 days from flowering in the concentration of the hexose monophosphate fraction; this may

PHYSIOLOGY OF PEA FRUITS

V. PHOSPHATE COMPOUNDS IN THE DEVELOPING SEED

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[Manuscript received June 14, 1957]

Summary

The changes in concentration of total phosphorus, total acid-soluble phosphorus, inorganic phosphorus, hexose phosphates, and adenosine pyrophosphates were measured in maturing seed of the pea (*Pisum sativum* L.) at different times from flowering.

The decrease in the value of the ratio inorganic phosphate/hexose monophosphate measured in the extracts of the seed preceded by some days the increase in the rate of starch synthesis. The rate of synthesis of starch in the seed was not closely related to the ratio inorganic phosphate/glucose 1-phosphate.

The accumulation of starch after 23 days from flowering was accompanied by decrease in concentration of hexose monophosphate. This decrease, presumably a direct consequence of the starch formation, would explain the accompanying loss of sucrose due to a reversal of the synthetic reactions in which it is formed.

The concentration of the phosphorus compounds did not appear to be closely related to the rate of protein synthesis or of respiration. The rate of respiration was not related to the ratio adenosine diphosphate/adenosine triphosphate.

Adenosine diphosphate, adenosine triphosphate, uridine triphosphate, and guanosine triphosphate were identified by paper chromatography in extracts from fresh pea seed.

I. INTRODUCTION

Previous workers have investigated changes in the concentration of carbohydrate fractions (Bisson and Jones 1932; McKee, Robertson, and Lee 1955; Danielson 1956; Turner and Turner 1957; Turner, Turner, and Lee 1957), protein and non-protein nitrogen (Bisson and Jones 1932; McKee, Robertson, and Lee 1955; McKee, Nestel, and Robertson 1955), inorganic and total phosphorus (McKee, Robertson, and Lee 1955), and in the rate of respiration (McKee, Robertson, and Lee 1955) in the maturing pea seed.

In the present paper, the concentrations of hexose phosphates, adenosine pyrophosphates, inorganic, total acid-soluble, and total phosphorus are reported in seeds sampled in two consecutive seasons, 1954 and 1955. The aim of the experiment was to look for correlations between changes in the concentration of compounds containing phosphorus and (1) changes in concentration of starch, sucrose, and protein nitrogen, and (2) changes in the rate of respiration.

In particular, it was intended to examine the hypothesis that the ratio adenosine diphosphate (ADP)/adenosine triphosphate (ATP), or the concentration of

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inorganic phosphate, limits the rate of respiration of tissue (Lardy 1952; Chance and Williams 1956). In experiments on the Pasteur effect in fresh pea seeds, Rowan, Seaman, and Turner (1956) have shown that the ratio ADP/ATP or the concentration of inorganic phosphate could limit the rate of aerobic respiration.

The mean seed mass and concentrations of starch reported here are those measured in replicate samples of seeds by Turner and Turner (1957), and those of sucrose by Turner, Turner, and Lee (1957).

II. MATERIALS AND METHODS

(a) *Sampling and Drying of Seed*

Seeds were first sampled at 12 days from full blossom; sampling continued at 2–5-day intervals until 30 days in 1954 and until 40 days in 1955. Turner and Turner (1957) have described the harvesting and drying of the seeds.

(b) *The Estimation of Phosphorus Fractions*

Samples containing 6–15 g of fresh seed were extracted by grinding with cold 1.5N HClO₄ and acid-washed sand, using a pestle and mortar (Rowan, Seaman, and Turner 1956; Rowan, unpublished data). This extract was used for the analysis of all acid-soluble fractions.

(i) *Inorganic Phosphorus*.—The method of Weil-Malherbe and Green (1951) was used.

(ii) *Total Acid-soluble Phosphorus*.—The method of Allen (1940) was used.

(iii) *Total Phosphorus*.—The dried seed was ground and samples of the powder analysed by the method of Berenblum and Chain (1938).

(iv) *Fructose 1,6-diphosphate (HDP), Hexose Monophosphate (HMP), ATP, and ADP*.—The enzymic method of Slater (1953) as modified by Rowan (1955 and unpublished data) was used. The concentration of hexose monophosphate measured was the sum of the concentrations of glucose 1-phosphate, glucose 6-phosphate, and fructose 6-phosphate.

(c) *Protein and Non-protein Nitrogen*

Total nitrogen was determined on dried material by a modified micro-Kjeldahl method (Turner 1949). Protein nitrogen was determined after extraction of the same material with 75 per cent. (v/v) ethanol, and soluble nitrogen calculated by difference.

(d) *Respiration Rate*

The rate of output of carbon dioxide by samples of pea seed (10–20 g) was measured in air at 25°C by the Pettenkofer method (Turner 1949; McKee, Robertson, and Lee 1955). Rowan, Seaman, and Turner (1956) have shown that in mature seed at this temperature, both the seed coat and cotyledons provide a slight resistance to diffusion, and it was probable that a small part of the carbon dioxide was formed in anaerobic fermentation.

TABLE 1
CHANGES IN FRACTIONS OF PHOSPHORUS AND IN PHOSPHORYLATED COMPOUNDS DURING THE DEVELOPMENT OF SEEDS, 1954

Days from Full Blossom	Mean Seed Mass (g)	No. of μ Moles/Gram Fresh Weight of:							Ratio ADP/ATP
		Inorganic Phosphorus	Total Acid-soluble Phosphorus	Unidentified Phosphorus	Hexose Mono-phosphate	Hexose Diphosphate	Adenosine Diphosphate (ADP)	Adenosine Triphosphate (ATP)	
12	0.033	10.6	19.5	6.1	0.846	0.149	0.198	0.374	0.53
15	0.082	12.1	18.6	3.6	0.944	0.179	0.243	0.291	0.84
17	0.119	11.4	18.4	2.1	3.154	0.226	0.131	0.356	0.44
19	0.179	10.4	16.9	3.0	6.328	0.278	0.328	0.392	0.83
21	0.323	8.3	15.8	0	6.242	0.290	0.471	0.406	1.19
24	0.380	8.2	20.9	5.8	2.244	0.536	0.542	0.849	0.63
28	0.369	3.8	27.0	18.0	1.027	0.426	0.725	0.605	1.09
30	0.400	3.3	28.1	20.0	1.168	0.335	0.358	0.743	0.48

III. RESULTS

Most of the results discussed in this paper are those from the longer experiment of 1955. The results from the shorter experiment of 1954 are given in Table 1 and are discussed only where they differ from those of 1955 or where the measurements were not made in 1955. The concentrations of metabolites are expressed both per seed and per gram fresh weight. Expressing concentrations per water content of seed (Danielson 1956) does not alter the conclusions to be drawn from the results.

(a) *Mean Seed Mass*

The mean seed mass of each sample is shown in Table 2.

TABLE 2
MEAN SEED MASS WITH TIME FROM FULL BLOSSOM IN 1955

Pick No.	Days from Full Blossom	Mean Seed Mass (g)	Pick No.	Days from Full Blossom	Mean Seed Mass (g)
1	12	0.034	7	25	0.504
2	15	0.114	8	27	0.605
3	17	0.174	9	30	0.601
4	19	0.216	10	33	0.599
5	21	0.335	11	36	0.558
6	23	0.450	12	40	0.412

(b) *Phosphate Esters*

(i) *Hexose Monophosphate*.—The concentration of hexose monophosphate per gram fresh weight increased sharply after 17 days (Fig. 1(a)). The concentration reached at 23 days was double that measured in the first picks. It then decreased until 30 days and increased again to a second maximum at 36 days. The first rise found in the experiment of 1954 was to six times the initial concentration (Table 1).

(ii) *Hexose Diphosphate*.—The concentration of hexose diphosphate per gram fresh weight remained approximately constant throughout the experiment (Fig. 1(a)).

(c) *Nucleotides*

(i) *Reactive Phosphate*.—The concentration of reactive phosphate ($\sim P$) is the sum of the β and γ phosphate groups of ADP and ATP, plus the γ phosphate group of uridine triphosphate (UTP) and guanosine triphosphate (GTP). It will be shown below that UTP and GTP are present in fresh pea seed in small concentration. Figure 1(a) shows that the concentration of reactive phosphate remained approximately constant between 12 and 17 days, rising to double the initial concentration at 27 days.

(ii) *ATP and ADP*.—Estimations of ATP and ADP are available on the samples of seed harvested in 1954 only. No precautions were taken to reduce interference by UTP and GTP present in the extracts (Rowan, unpublished data). As shown

below, the concentrations of UTP and GTP in pea seed are much lower than that of ATP, and the correction required is not likely to be large. The concentrations of

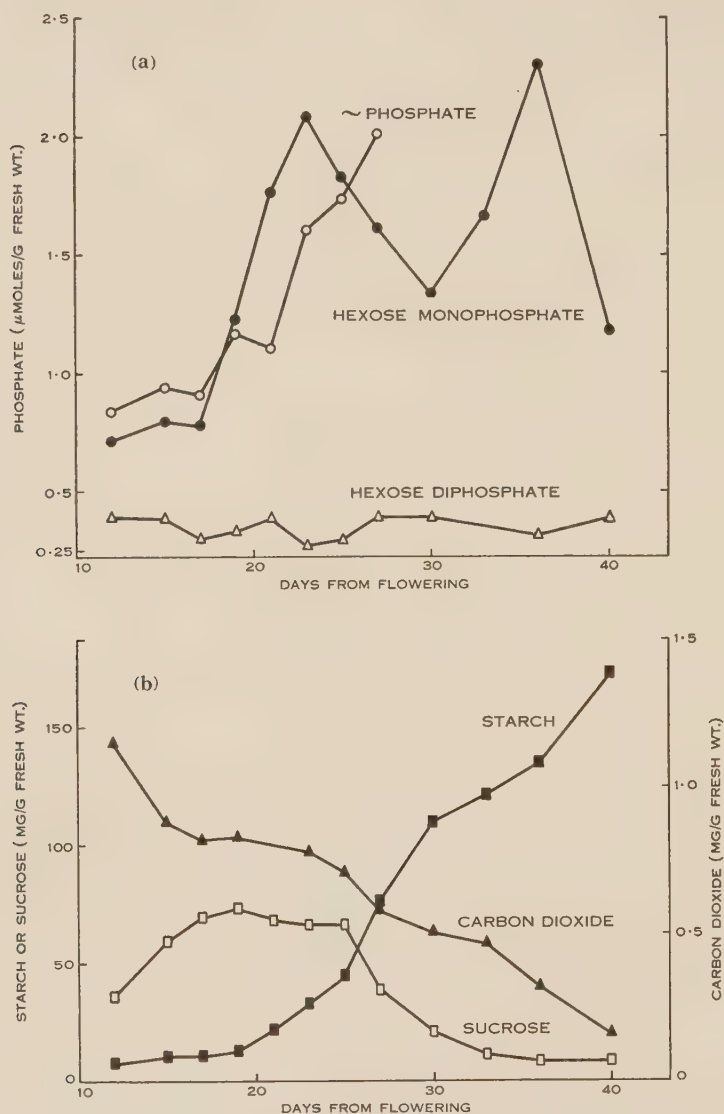


Fig. 1.—Changes in the concentration of (a) hexose monophosphate, hexose diphosphate, and reactive phosphate per gram fresh weight, and (b) starch and sucrose and in the rate of respiration per gram fresh weight with time from flowering.

both ADP and ATP tended to rise throughout the experiment, and no significant changes in the ratio ADP/ATP were observed (Table 1).

(iii) *UTP and GTP*.—UTP and GTP react in the enzyme system used to measure the concentrations of adenosine pyrophosphates (Rowan, unpublished data),

and, although it was not possible to extract nucleotides from the samples of seed used for the experiments described here, it was considered important to see if UTP and GTP were present in fresh seed.

Nucleotides were extracted from a sample of fresh seed by the method of Albaum, Ogur, and Hirshfeld (1950) and examined by the methods described by

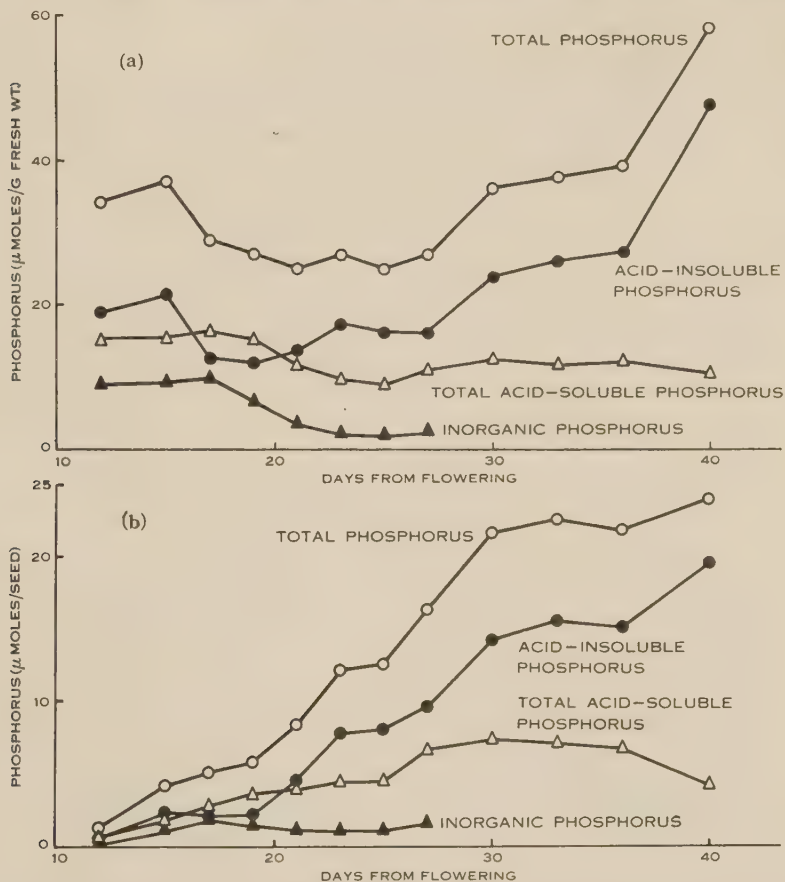


Fig. 2.—Changes in the concentration of total, acid-insoluble, total acid-soluble, and inorganic phosphorus (a) per gram fresh weight and (b) per seed with time from flowering.

Rowan (1957). The chromatogram on Plate 1 shows that GTP was present in addition to the nucleotides already found in young pea seedlings (Rowan 1957, Fig. 3). The lines containing ATP, ADP, UTP, and GTP were identified by running spots of authentic compounds on chromatograms. Samples of UTP and GTP were obtained by eluting the chromatograms and the γ phosphate groups shown to analyse as reactive phosphate in the method of Slater (1953).

The intensity of the lines of UTP and GTP on the chromatogram shown in Plate 1 is much less than that of ATP or ADP. Though extraction of nucleotides

by the method of Albaum *et al.* (1950) is not intended to be quantitative, it seems probable that the interference by UTP and GTP in the estimation of adenosine pyrophosphates will not be large.

(d) *Fractions of Phosphorus*

The changes in concentration of these fractions are shown in Figure 2. The main points in these results were:

(i) *Total Phosphorus*.—The amount per seed increased until the end of the experiment, though the rate of increase was small after 30 days. The concentration expressed as per gram fresh weight decreased until 25 days and then increased until the end of the experiment.

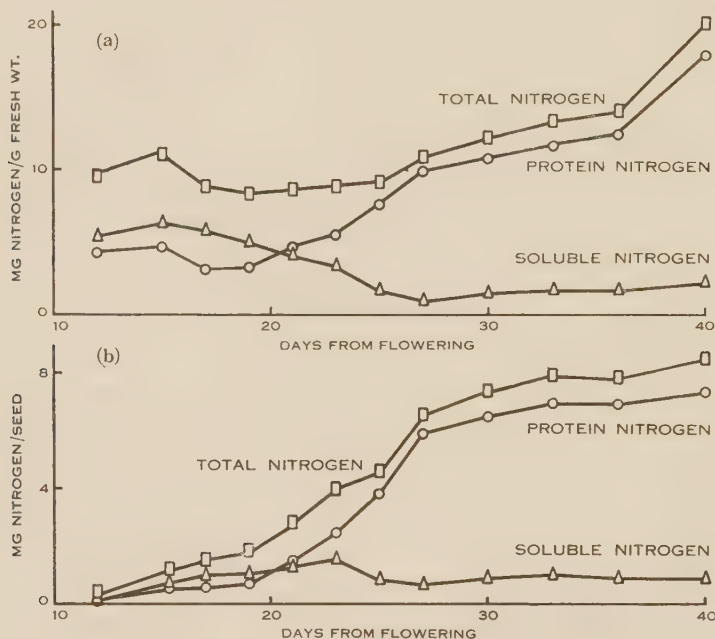


Fig. 3.—Changes in the concentration of total, protein, and soluble nitrogen (a) per gram fresh weight and (b) per seed with time from flowering.

(ii) *Acid-insoluble Phosphorus*.—The concentration per seed increased until the end of the experiment, when it was about 80 per cent. of the total phosphorus.

(iii) *Total Acid-soluble Phosphorus*.—The concentration changed in the same manner as total phosphorus until 30 days, when total acid-soluble phosphorus expressed both as per seed and per gram fresh weight decreased.

(iv) *Inorganic Phosphorus*.—The concentration per gram fresh weight fell between 17 and 25 days (Fig. 2(a)). The fall was sufficiently marked to be apparent when the concentration was expressed as per seed (Fig. 2(b)) and was thus similar to that found by McKee, Robertson, and Lee (1955). Reliable figures are not available after 27 days.

(e) Fractions of Nitrogen

The changes in protein and soluble nitrogen (Fig. 3) were similar to those found by McKee, Robertson, and Lee (1955). After 19 days, the concentration of protein nitrogen increased above that of soluble nitrogen and the rate of movement of nitrogen into the seed and the rate of synthesis of protein increased.

(f) Respiration

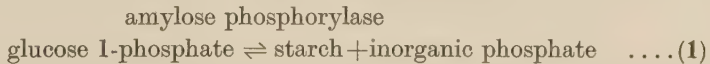
The changes in rate of respiration (Fig. 1(b)) were similar to those found by McKee, Robertson, and Lee (1955). The rate per fresh weight decreased throughout the experiment, except for a slight rise at 19 days.

IV. DISCUSSION

Although the fresh and dry weight of seeds from the harvest of 1954 were lower than those of 1955 (Turner and Turner 1957), the amount of the phosphate compounds per gram fresh weight did not differ significantly, except for the maximum concentration of hexose monophosphate, which in 1954 was three times higher than in 1955.

(a) The Relationship of Inorganic Phosphate and Hexose Monophosphate to Starch Synthesis

In vitro, the rate of synthesis of starch by the reaction



is independent of the concentration of starch formed but is a function of pH and the ratio inorganic phosphate/glucose 1-phosphate (Hanes and Maskell 1942). At equilibrium, this ratio falls from 11 at pH 5 to 2 at pH 7.

The rate of synthesis of starch in our experiment was calculated from tangents to a curve of best fit drawn through a graph of the concentration of starch per fresh weight plotted against time from full blossom (Fig. 1(b)). The ratio inorganic phosphate/hexose monophosphate decreased from approximately 13 to 2 some days before the rate of synthesis of starch increased to a maximum at 25 days (Fig. 4). Therefore, if we assume that glucose 1-phosphate was a constant fraction of the concentration of hexose monophosphate measured, it is unlikely that the rate of synthesis of starch at different stages of maturity of the seed was determined solely by the overall ratio inorganic phosphate/glucose 1-phosphate in the tissue.

Turner and Turner (1957) have shown a linear relationship between rate of synthesis of starch and the concentration of amylose phosphorylase, and it appears that the concentration of this enzyme, rather than the fall in the ratio inorganic phosphate/glucose 1-phosphate underlies the increase in the rate of synthesis of starch as the seed matures. The decrease in concentration of hexose monophosphate which occurred after 23 days is consistent with the increasing rate of utilization of hexose monophosphate in starch synthesis. Similarly, the rise in concentration of hexose monophosphate after 30 days is consistent with the decrease in rate of starch synthesis (Fig. 1(b)).

Figure 4 shows that some synthesis of starch occurred while the ratio inorganic phosphate/hexose monophosphate was above 11. Even if all the hexose monophosphate were glucose 1-phosphate and the pH at the site of synthesis were 5, no synthesis of starch would have occurred under these conditions *in vitro* (see eqn. (1)). Therefore, it is probable that the ratio inorganic phosphate/hexose monophosphate measured was not that at the site of synthesis of starch *in vivo*.

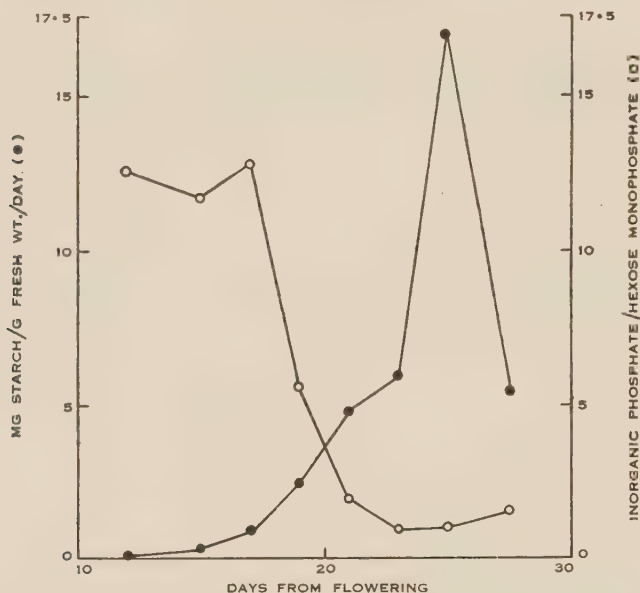
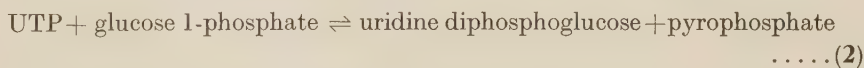


Fig. 4.—Changes in the rate of starch synthesis and in the ratio inorganic phosphate/hexose monophosphate with time from flowering.

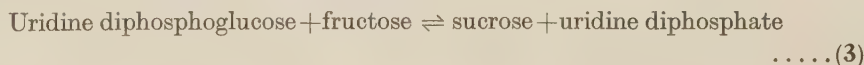
(b) *The Relationship of Hexose Monophosphate to Sucrose*

As mentioned already, the concentration of sucrose in the young seed was higher than that of starch, but subsequently the concentration of sucrose decreased, and carbohydrate, arriving at the seed by translocation as a sugar (Turner and Turner 1957), was stored as starch. In the present experiment, the decrease in concentration of sucrose occurred after 25 days (Fig. 1(b)), when the rate of synthesis of starch was at a maximum (Fig. 4).

Turner (1953, 1954), Cardini, Leloir, and Chiriboga (1955), and Turner and Turner (unpublished data) have shown that the reversible reactions shown in equations (2) and (3) occur in pea seed:



(Kalckar and Cutolo 1952; Munch-Petersen *et al.* 1953).



(Leloir and Cardini 1953).

The fall in concentration of hexose monophosphate between 23 and 30 days due to the rapid synthesis of starch could stimulate the reverse reactions of equations (2) and (3), and thus tend to bring about the conversion of sucrose to starch by this pathway in the later stages of development of the seed.

(c) *Phosphorus Fractions*

The important conclusions to be drawn from the changes in concentration of the fractions of phosphorus are as follows:

(i) Although the amount of total phosphorus per seed increased throughout the experiment (Fig. 2(b)) the movement of phosphorus into the seed did not keep pace with the increase in fresh weight, and the concentration per gram fresh weight decreased until 25 days (Fig. 2(a)).

(ii) The percentage of inorganic phosphorus fell from 25 to 8 per cent. of total phosphorus between 12 and 25 days.

(iii) After 25 days, the concentration of total acid-soluble phosphorus per gram fresh weight increased slightly to the end of the experiment. In 1954, this rise was more marked, and was caused by an increase in unidentified organic phosphorus (Table 1). It is probable that much of this unidentified phosphorus was phytin, which Fowler (1956) has isolated from pea flour.

(iv) After 25 days, the proportion of phosphorus found in the acid-insoluble fraction increased rapidly (Fig. 2(b)). A part of this increase was at the expense of the acid-soluble fraction.

(d) *Protein Synthesis*

The amount of protein nitrogen per seed increased rapidly after 19 days (Fig. 3); until this time, the net rate of synthesis of protein was low, and the concentration expressed per gram fresh weight decreased. The concentration of reactive phosphate increased at the time when the rate of protein synthesis increased (Figs. 1 and 3).

Webster (1954, 1955, 1956) has shown that ATP stimulated the incorporation of amino acids into protein in homogenates of plant tissue, and it is thus possible that the concentration of ATP is a factor governing the rate of synthesis of protein in pea seed. However, too few observations are available for a sensitive measurement of the correlation between the two variables. It is also possible that the concentration of metabolites reflected the concentration of the enzymic systems in which they were formed. Inspection of Figures 1(a) and 1(b) support this, for any correlation calculated between the rate of synthesis of protein and the concentration of hexose monophosphate or starch would be as close as that with reactive phosphate.

(e) *Respiration Rate*

The rate of respiration per gram fresh weight decreased throughout the experiment in a manner similar to that found by McKee, Robertson, and Lee (1955). The concentration per gram fresh weight of the phosphate compounds and fractions analysed did not follow closely the plot of the rate of respiration (Figs. 1(a) and 1(b)),

though the decrease in the concentration of total phosphorus, total acid-soluble phosphorus, and inorganic phosphorus between 15 and 25 days corresponded approximately to the fall in the rate of respiration.

The fall in respiration rate was not reflected in any change in the concentration of hexose diphosphate, which remained constant throughout the period. In spite of the increase in synthetic rate after 19 days and the fall in respiration rate, the concentration of reactive phosphate increased, showing that energy production by respiration did not limit phosphorylation at any stage.

The ratio ADP/ATP, measured in 1954, fluctuated between 0.44 and 1.19 (Table 1), but did not appear to follow the drift in the rate of respiration. Also, throughout the preliminary experiment on the variety Shaster (Rowan 1955), the ratio remained constant. Therefore, it appears that the rate of respiration of the tissue of the seed is limited by a factor or factors other than those investigated in this experiment.

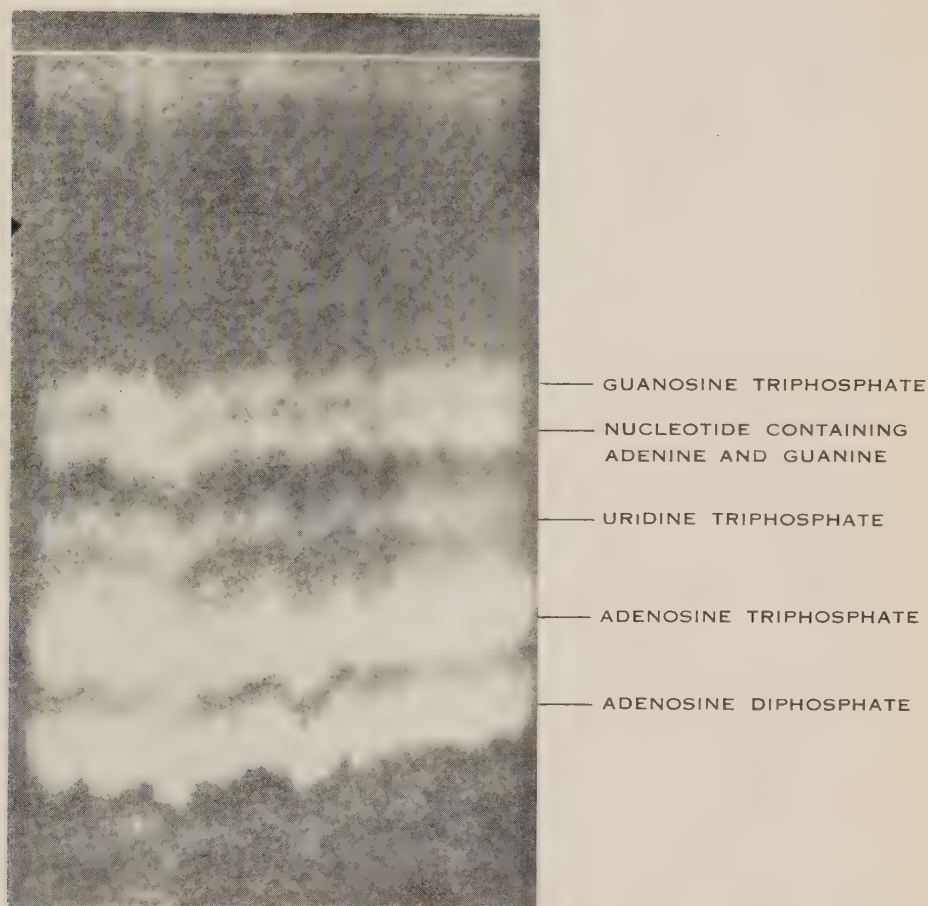
V. ACKNOWLEDGMENTS

We would like to thank Mr. L. J. Lynch and Mr. R. S. Mitchell for their advice, Hawkesbury Agricultural College for the supply of peas, Dr. R. N. Robertson and Dr. J. F. Turner for helpful discussion, Professor R. K. Morton and Dr. F. J. R. Hird for criticism of the manuscript, Mr. G. G. Coote for advice on the analysis of the data, Mr. D. E. Seaman and Mr. N. F. Tobin for preparing some of the seed extracts, and Mr. M. F. Clayton, Mr. R. W. Hinde, Mr. J. B. Lee, Miss P. D. Archer, and Miss J. A. Talbot for technical assistance.

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PHYSIOLOGY OF PEA FRUITS. V



Chromatogram of an extract of nucleotides from fresh pea seed run as a line in *n*-propanol-ammonia (sp. gr. 0.880)-water (60 : 30 : 10 v/v) (Hanes and Isherwood 1949). The chromatogram was photographed with ultraviolet light by a modification (Rowan 1957) of the method of Markham (1955). Running time 40 hr on acid-washed Whatman No. 3 filter paper.

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THE EFFECT OF AUXINS ON THE BINDING OF PECTIN METHYLESTERASE TO CELL WALL PREPARATIONS

By K. T. GLASZIOU*

[Manuscript received June 18, 1957]

Summary

It is shown that the plant auxins 3-indolylacetic acid, 2,4-dichlorophenoxyacetic acid, and α -naphthalene acetic acid are effective in binding pectin methylesterase (PME) to cell wall preparations from tobacco pith and tubers of the Jerusalem artichoke.

Each auxin has an optimum concentration for activity. The activity curves are consistent with the hypothesis that the auxin binds PME to cell wall receptor sites by an adsorption mechanism which can be described by the Langmuir adsorption isotherm.

It is suggested that the auxin-mediated binding of PME could control extension growth by controlling the extent of esterification of the pectic substances of the cell wall.

I. INTRODUCTION

In an earlier paper (Glasziou 1957) it was suggested that auxins may exert their effect by helping to bind enzymes of cell wall metabolism to receptor sites within the cell. A technique was described by which a pectin methylesterase (PME)-cell wall complex could be split in salt solution and subsequently recombined in the presence of 3-indolylacetic acid (IAA), with an optimal effect at about 10^{-10} M IAA.

The work presented in this paper extends the observations on the binding of PME to cell wall in the presence of IAA, and includes observations with the growth substances 2,4-dichlorophenoxyacetic acid (2,4-D) and α -naphthalene acetic acid (NAA). The effect of the growth substances is shown to occur in preparations from top internodes of young, actively growing tobacco plants and from the tubers of Jerusalem artichokes, as well as from mature stems of tobacco plants. Some observations on the effects of calcium ions and ethylenediaminetetra-acetic acid (EDTA) are presented, and the possible relationship between the effect of auxins on the binding of PME and their effect on the growth rate of *Avena* coleoptiles is discussed.

II. METHODS AND MATERIALS

The method described previously (Glasziou 1957) for separating PME from the wall fraction and for reconstituting a wall-PME complex has been followed with minor alterations. In outline the method consisted of isolating a wall-PME complex by differential centrifugation of a tissue homogenate and then splitting the complex with 10 per cent. sodium chloride. The solubilized PME was removed from the wall fraction by filtration and dialysed; the wall fraction was washed extensively to

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remove sodium chloride and residual PME. The two components were recombined by incubating with IAA at one of several concentrations. Uncombined PME and excess IAA were removed by filtration and washing, and the PME activity of the wall fraction was assayed. This technique has been modified by (i) including EDTA at a final concentration of $1 \times 10^{-3} \text{M}$ in the buffer for homogenization of the tissue, (ii) increasing the period used during dialysis of the PME solution to about 18 hr, and (iii) raising the pH for incubation of the wall fraction, PME, and auxin to 7.0.

It has become apparent that the method which yielded consistent results with tissue from mature tobacco stems which had flowered required modification to obtain active preparations from some other tissues. Marked differences in preparations from different plants or tubers have been observed, particularly in the presence of a component in the wall preparation to which PME is adsorbed in the absence of added growth substance. This non-specific adsorption usually decreased with time of storage of wall fraction in the cold, but in one experiment it increased.

Enzyme assay.— The method previously used was modified to permit assay of smaller amounts of PME. The assay mixture consisted of 25 ml 0.5 per cent. citrus pectin (purified by the method of Kertesz 1957), and 1.5 ml 1.0M CaCl_2 . The pectin solution was prepared freshly each day by being dispersed in a Waring Blendor, filtered, and neutralized immediately prior to use. After addition of the enzyme, the assay system was adjusted to pH 7.5 (glass electrode) with 0.005N NaOH, and then one drop extra of alkali added to raise the pH to about 7.7. A stop-watch was started when the pH returned to 7.5. The pH was continually adjusted to 7.0–7.5 during the course of the assay. The end-point was determined in the same way as the starting point, i.e. the pH was adjusted to 7.7 and the time taken when the pH returned to 7.5. The time interval, which varied between experiments (depending on the activity of the enzyme), was usually 10 min, but occasionally longer. With reasonable care and close attention to the condition of the pH meter and electrodes, the method gives results which are reproducible to within 0.025 ml of the titrating alkali.

III. RESULTS

Figure 1(a) shows the effect of IAA on the formation of a wall-PME complex in a preparation from the pith of tobacco plants which had flowered. In Figure 1(b) the reciprocal plot of data taken from the curve of Figure 1(a) is shown together with the calculated auxin concentration which gives half the maximum adsorption of PME to the cell wall (K_{auxin}). This is similar in derivation to the growth constant of Foster, McRae, and Bonner (1952), which, as they point out, has analogies to a Michaelis constant.

Data for reciprocal plots given in this paper have been obtained from the curves fitted to the experimentally determined points. This procedure was necessary because of large differences in auxin concentrations required to give significant differences in the activity of the wall-PME complex. The justification for such a procedure was that, in seven experiments, a straight line could be drawn as line of best fit in the reciprocal plots and each extrapolated line cut the vertical axis. The shape of the curve proximal to the vertical axis has important theoretical

implications which are discussed later. A value of about 10^{-10}M was consistently obtained (see Fig. 1(a)) for the optimal concentration of IAA for the binding of PME to wall preparations from tobacco plants and from artichoke tubers, but in one preparation from tobacco plants, 2–3 ft tall, the optimal concentration was between 10^{-6} and 10^{-7}M . This was the highest value obtained in any experiment.

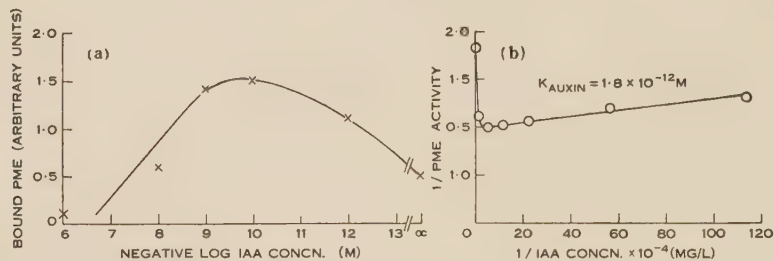


Fig. 1.—(a) Effect of IAA on the binding of PME to a cell wall preparation from the pith of tobacco plants which had flowered. (b) Reciprocal plot taken from the smooth curve of (a) from 5×10^{-9} to $5 \times 10^{-12}\text{M}$ IAA.

Many preparations from tobacco plants 3–5 ft tall and from artichoke tubers showed high non-specific adsorption of the enzyme (adsorption of PME to the wall fraction in the absence of added auxin) which tended to mask the effects of added auxin (Fig. 2). These curves are apparently composite, reflecting both the

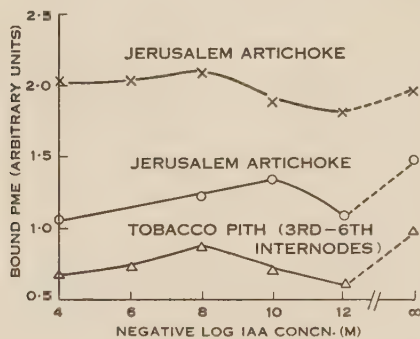


Fig. 2

Fig. 2.—Effect of IAA on the binding of PME to cell wall preparations which show high non-specific adsorption of PME.

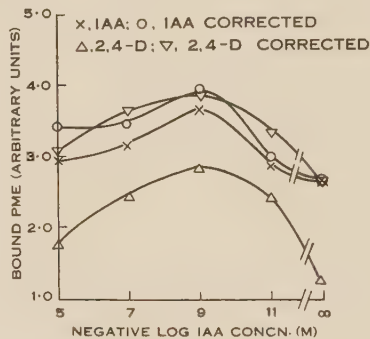


Fig. 3

Fig. 3.—Effect of IAA and 2,4-D on the binding of PME to a cell wall preparation from the pith of the top internodes of young tobacco plants (see text for explanation of "corrected").

effect of auxin and the non-specific adsorption. It can be seen in Figure 2 that auxin appeared to have a depressing effect on the non-specific adsorption which may be due to the formation of a PME-auxin complex of different adsorption characteristics.

Non-specific adsorption sometimes decreased with time, and when this occurred it was necessary to correct the data to account for changes taking place during the course of the experiment. In Figure 3, the corrected and uncorrected curves are plotted for the effects of IAA and 2,4-D on a preparation from tobacco pith in

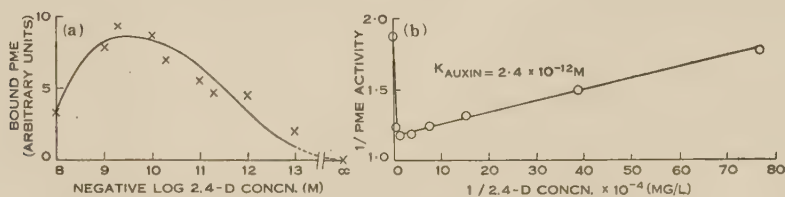


Fig. 4.—(a) Effect of 2,4-D on the binding of PME to an EDTA-treated cell wall preparation from tubers of the Jerusalem artichoke. (b) Reciprocal plot taken from the smooth curve of (a) from 5×10^{-9} to $5 \times 10^{-12} M$ 2,4-D.

which the non-specific adsorption decreased with time. The necessity for these corrections could be avoided if all treatments and assays were carried out simultaneously, when the non-specific adsorption would be more nearly constant in all treatments.

TABLE 1

EFFECT OF 2,4-D AND CALCIUM IONS ON THE BINDING OF PECTIN METHYLESTERASE (PME) TO CELL WALL CONSTITUENTS OF ARTICHOKE TUBERS

The complete system contained 4.0 ml wall suspension, 4.0 ml PME solution, and additions as shown to give a final volume of 10.0 ml. After incubation for 30 min at 22°C the residues were filtered, washed, and the enzyme activity assayed

Experiment No.	2,4-D Concn. (M)	Calcium Ion Concn. (M)	PME Activity (arbitrary units)
1	—	—	0.24
2	1×10^{-7}	—	0.84
3	1×10^{-9}	—	1.00
4	1×10^{-11}	—	1.26
5	—	1×10^{-2}	3.52
6	1×10^{-7}	1×10^{-2}	2.34
7	1×10^{-9}	1×10^{-2}	3.02
8	1×10^{-11}	1×10^{-2}	3.12

The amount of non-specific adsorption varied with the experimental material, and was sometimes negligible. The effect of 2,4-D on a preparation from artichoke tubers which showed very low non-specific adsorption is shown in Figure 4(a), and in Figure 4(b) the reciprocal plot for 2,4-D concentration between 5×10^{-9} and $5 \times 10^{-12} M$ is given. The artichokes for this experiment were freshly dug and EDTA was included in the buffer for homogenization of the tissue in the Waring Blender.

Similar preparations from the same batch of artichokes showed high non-specific adsorption after storing in damp sand in a cold room at 5°C for 1 week. Adamson and Adamson (1957) using the same artichokes found no lag period for expansion in the presence of auxins for tissue from freshly dug immature tubers, but that a lag period developed on storage at low temperature.

At least part of the non-specific adsorption of PME to cell wall preparations may be attributed to the presence of calcium ions. Table 1 gives the effects of 2, 4-D and calcium ions on the reconstitution of the PME-wall complex in the preparation from artichoke tubers, from which it can be seen that the addition of calcium ions at a concentration of $1 \times 10^{-3}M$ markedly increased the adsorption of PME to the wall fraction. The effect of calcium ions was greatest in the absence of added auxin. Nevertheless, there was a clear auxin effect superimposed on that due to calcium ions. These results compare with those shown in Figure 2 and are presented as further

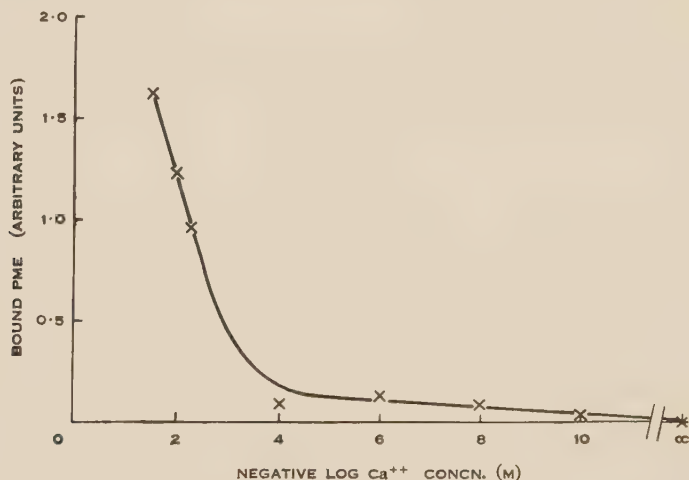


Fig. 5.—Effect of calcium ions on the binding of PME to an EDTA-treated cell wall preparation from tubers of the Jerusalem artichoke.

evidence that auxins alter the adsorption properties of PME, perhaps by the formation of a PME-auxin complex. The effect of varying concentrations of calcium ions on the adsorption of PME to a wall fraction from artichoke tubers is shown in Figure 5. Relatively little effect was observed at concentrations less than $1 \times 10^{-4}M$, but a very marked effect was observed at concentrations from about 1×10^{-3} to $3 \times 10^{-2}M$ Ca^{++} . This effect is not to be confused with the well-known effect of divalent cations on the activation of PME, a possibility guarded against by the extensive washing of the PME-wall complex before assay of the PME activity, and also by the presence of $5.7 \times 10^{-2}M$ Ca^{++} in the assay system.

That NAA has similar effects to IAA and 2,4-D has been demonstrated on preparations from both young tobacco plants and freshly dug artichoke tubers. In Figures 6(a) and 6(b) results for an experiment on tobacco pith from the top internodes of plants about 3–4 ft high and the reciprocal plot are given. In experiments with NAA the washing procedure, after reconstitution of the wall-PME com-

plex, was reduced to two washings with 5-ml portions of 0.001M phosphate buffer, pH 7.0. It is not known whether the higher K_{auxin} value for NAA as compared with those obtained for IAA and 2,4-D indicates that NAA has a lower affinity in the system for binding PME to the wall. A decision on these kinetic aspects must await purification of the system components.

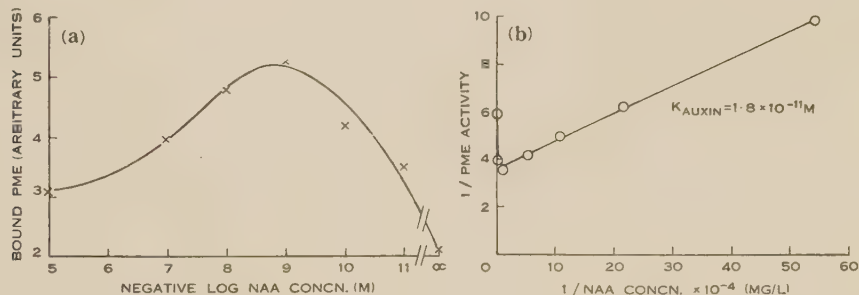


Fig. 6.—(a) Effect of NAA on the binding of PME to an EDTA-treated cell wall preparation from the top internodes of young tobacco plants. (b) Reciprocal plot taken from the smooth curve of (a) from 1×10^{-7} to 1×10^{-11} M NAA.

IV. DISCUSSION

It has been established that the plant growth substances IAA, 2,4-D, and NAA are effective in binding PME to cell wall preparations from mature stems of tobacco plants which had flowered, from top internodes of young rapidly growing tobacco plants, and from tubers of the Jerusalem artichoke. The optimal concentration of growth substance is of the order of 10^{-10} M, and there is a falling-off of the effect at higher and lower concentrations. It can reasonably be presumed that the effect of higher concentrations is an inhibition of the binding of the enzyme rather than a lowering of its activity, as the addition of IAA, 2,4-D, or NAA to the assay system at concentrations above the optimal has no effect on the activity of either the soluble enzyme or the bound enzyme.

The accuracy with which the effect of growth substances on the binding of PME to the cell wall can be measured is not yet sufficiently high to offer conclusive proof that mathematical treatment in terms similar to classical enzyme kinetics would be valid. However, in all cases where sufficient data have been obtained, reciprocal plots of the PME activity of the wall-enzyme complex against the auxin concentrations gave a linear relationship at low concentrations of the auxin and at high concentrations the curve rose sharply. Thus it is a reasonable assumption, as a basis for discussion, that these results can be treated in a similar manner to that used by Foster *et al.* (1952) for analysing the effect of auxin on coleoptile growth.

Relation to other Work

Heyn (1931) suggested that extension growth under the influence of auxin involves an increase in cell wall plasticity, and Kerr (1951), in considering the properties of cellulose and pectic substances, suggested that the properties of the

primary wall could be explained on the assumption that protopectin forms a continuous phase and the cellulose microfibrils form a discontinuous phase. Bennet-Clark (1955) considered that the pectins of the walls have maximum extensibility when the carboxyl groups are converted to methyl esters, and Ordin, Cleland, and Bonner (1955) have demonstrated that auxin influences the incorporation of methionine- ^{14}C , labelled in the methyl group, into the hot-water-soluble non-cellulosic fraction of the cell wall.

Recent electron microscope studies of the microfibrillar pattern of *Avena* coleoptile parenchyma cells (Wardrop 1955, 1956) indicate that during extension growth the wall expands evenly and that new, transversely orientated microfibrils are deposited as growth proceeds. The subsequent disorientation of the microfibrils takes place in a manner consistent with a stretching of the wall, and this may be facilitated by changes in the non-cellulosic cell wall constituents.

The work of Foster *et al.* (1952) and McRae, Foster, and Bonner (1953) has shown that, under certain rigidly defined experimental conditions, the concentration of added growth substance may become rate limiting for the growth of the *Avena* coleoptile. Under such conditions, the growth response was related to the concentration of the growth substance in a manner similar to the relation of rate of enzyme reactions to concentration of substrates for certain enzymes which are inhibited by high concentrations of their substrates. The growth substance was considered to bind at two points to a cellular component to form a growth-active complex. At high auxin concentrations, competition by individual auxin molecules for the attachment sites on the cellular component gave rise to a complex in which two auxin molecules bound, one at each point, were considered to inhibit growth. When the reciprocal of the growth rate was plotted against the reciprocal of the auxin concentration, a straight line was obtained up to the optimal concentration after which the curve rose steeply. The reciprocal plots for the binding of PME to a wall component in the presence of auxins give the same type of curve. It is pertinent to consider whether these two auxin-mediated reactions are actually the same reaction.

It has been proposed previously (Glasziou 1957) that the activity of PME could control the extent of esterification of the pectic substances and so control their elastic and plastic extension properties. To relate the auxin-mediated growth reaction in coleoptiles and the auxin-mediated binding of PME to the cell wall, it is necessary to assume that growth under the conditions used by Bonner's group was inversely related to PME activity, which in turn was related to the concentration of auxin, being progressively decreased by concentrations up to the optimal and increased at supraoptimal concentrations.

Foster *et al.* (1952) pointed out the analogy of their treatment to the Michaelis-Menten hypothesis for enzyme action which assumes the formation of an intermediate enzyme-substrate complex, the rate of the reaction being governed by the number of adsorbed substrate molecules. However, this relationship can also be derived from the Langmuir adsorption isotherm. That the relationship between growth and auxin concentration, and the adsorption of PME to the cell wall can be described by a similar mathematical treatment as that for an enzyme and substrate implies only that all are examples of a type of adsorption phenomenon. The Michaelis

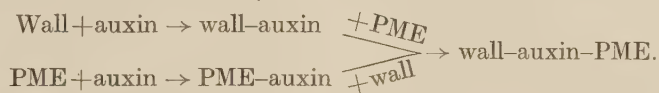
constant, although calculated in the same way, does not have the same significance in the different reaction systems.

Results for the binding of PME in the presence of auxins have been obtained with tobacco pith and the tubers of the Jerusalem artichoke. Adamson and Adamson (unpublished data) have observed that disks from Jerusalem artichoke tubers expand in the presence of auxins (IAA, 2,4-D, and NAA), the rate of expansion (after the lag period) being linear with time over periods of up to 48 hr, and over a range of auxin concentrations above the optimal. Reciprocal plots of the growth rate against auxin concentration give the same kind of relationship as for the *Avena* coleoptile.

A tentative mechanism by which the auxin-mediated growth of plant tissues may be related to the binding of PME are as follows: Firstly, the steady-state extension growth is dependent on the extent of methylation of the pectic substances of the cell wall; secondly, the reactions involved in the synthesis and methylation of pectates may be described generally as:



and, thirdly, the activity of PME is assumed to be controlled by auxin-mediated binding in the following manner:



If the wall-auxin-PME complex is considered to be the ineffective form of PME in the cell, methylation of pectates will be at a maximum when there is maximum binding of PME to the wall sites. At high concentrations of auxin, the decreased concentration of free PME and wall sites would limit the formation of the wall-auxin-PME complex. The enzymatic activity of PME and PME-auxin is the same *in vitro* and may be presumed to be the same *in vivo*. Given these relationships, it is feasible that the rate-limiting reaction for extension growth could be the auxin-mediated binding of PME.

An apparent anomaly in this interpretation is that the optimal concentrations of auxins for coleoptile growth measured by Bonner's group were much higher than the optimal values for the binding of PME to the wall sites. The values for PME-binding were obtained in a simple aqueous system in which the activities (in the physicochemical sense) of the components would not be very different from their actual molar concentrations. However, in the cell the activities of these components are likely to be very different from their molar concentrations due to the complex nature of the cytoplasm, and so the anomaly may not be as serious as it first appears.

The non-specific binding of PME to components of the wall fraction has been partially attributed to the effect of calcium ions, but not all non-specific binding can be explained in this way. Such binding may be either an artifact of no significance in the intact cell or some complementary mechanism controlling PME activity. The effects of calcium ions on binding of PME are of interest because of the relationship between auxins and calcium ions on the extension growth of *Avena* coleoptiles

(Bennet-Clark 1955) and the effects of chelating agents (Heath and Clark 1956). Also Burström (1954) has observed that the effect of calcium ions in root growth resembles that of an anti-auxin, increasing the cell elongation and apparently antagonizing an auxin. Some aspects of these effects may be explained if the wall- Ca^{++} -PME complex is less effective in reducing the activity of PME than is the wall-auxin-PME complex. These relationships are complicated by the effect of calcium ions on the physical properties of pectates and the fact that calcium ions are powerful activators of PME.

The proposed mechanism by which the effect of auxin on the binding of PME could become manifested in cell elongation is only one of several which could be advanced. Further evidence is required on the effects of other auxin-like compounds (particularly competitive inhibitors of auxin action) on the adsorption of PME to the wall sites. However, it will be necessary to purify the components of the system to obtain conclusive results in experiments of this type. Work along these lines is now in progress and will be reported in a subsequent paper.

V. ACKNOWLEDGMENTS

The work described in this paper was undertaken as part of a joint research programme of The Colonial Sugar Refining Co. Ltd., the Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O., and the Botany School, University of Sydney. The author wishes to thank the management of the Company for permission to publish this paper; Dr. R. N. Robertson, C.S.I.R.O., for his interest and advice throughout the course of the work; and Professor Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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THE LAG PERIOD IN AUXIN-INDUCED EXPANSION OF STORAGE TISSUE AND COLEOPTILES

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[*Manuscript received June 19, 1957*]

Summary

Auxin caused a rapid shift in the point of incipient plasmolysis of a number of tissues which showed auxin-induced expansion. The plasmolysis shift was not caused by dilution of the cell sap and might be due to an effect of auxin on the adhesion between cytoplasm and cell wall.

The lag period in auxin-induced expansion of Jerusalem artichoke storage parenchyma depended on the physiological age of the tissue. Tissue from immature tubers had no lag period and the lag period increased with storage or maturation. A lag period is characteristic of stored or mature tissue only.

When expansion was reduced with mannitol solutions, a lag period was induced in coleoptile response to auxin. The lag period increased with time of pretreatment in isotonic mannitol and was inversely proportional to elongation occurring during pretreatment with a range of hypotonic concentrations.

It is concluded that the transition from expanding to non-expanding wall structure results in the development of a lag period and depends upon the balance between cell wall metabolism and turgor pressure.

I. INTRODUCTION

Auxin added to coleoptile tissue increases the rate of elongation, which then remains constant for several hours. The increased rate of elongation due to the presence of auxin is established with no observable delay, as indicated for example by Bonner and Foster (1955). On the other hand, with storage tissue there is a variable lag period between auxin addition and the subsequent auxin-induced expansion. This lag period was first shown by Reinders (1938) with potato and has been studied in Jerusalem artichoke by Hanson and Bonner (1955), who tentatively attribute the lag to the time taken for the tissue to synthesise an enzyme or enzymes essential to auxin-induced water uptake. The presence of a lag period in the auxin-induced expansion of storage tissues and its absence in elongating coleoptiles has been considered characteristic of the type of tissue.

This paper shows that there is no lag period in immature storage tissue of Jerusalem artichoke and that the development of a lag is related to tissue maturity. A lag period in elongation of coleoptiles can be induced by preventing expansion for short periods prior to auxin treatment. Auxins also cause a rapid shift in the point of incipient plasmolysis of a number of tissues which show auxin-induced expansion. It is suggested that there is no delay in auxin action intimately associated with the wall, but that the lag in auxin-induced expansion is related to the structural properties of the cell wall.

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II. MATERIALS AND METHODS

Storage parenchyma of Jerusalem artichoke (*Helianthus tuberosus*) tubers and coleoptiles of wheat (var. Federation) were used as experimental material. Slices of artichoke tuber 1 mm thick were cut, washed, and aerated in distilled water for at least 2 days before use. For each complete experiment the tissue was cut from a single tuber. Wheat seeds were soaked in shallow water for 1 hr, sown in damp sand, and grown in the dark at 25°C. Uniform coleoptiles were harvested at 85 hr from sowing. From each coleoptile, 10-mm sections were cut 3 mm below the apex. The sections were randomized and replicates of 10 sections per treatment were used. Except during cutting when the sections were in very dim light for about 1 min and measurement, which took *c.* 3 min, the sections were kept in the dark.

All solutions were buffered to pH 4.5 with 0.005M potassium phosphate. Solutions were stored at 5°C and renewed after 5 days.

The strength of the external solution causing plasmolysis in 50 per cent. of the cells was determined by microscopic observation in a range of mannitol solutions. In all treatments involving β -indolylacetic acid (IAA), the tissue was transferred direct from distilled water to the appropriate mannitol solution containing IAA.

The effect of IAA on artichoke tissue was determined by the change of wet weight with time. During experiments extending over several days, the solutions were aerated continuously and changed at least daily. Twelve randomly selected slices per sample were used in each treatment. Wet weight was determined using a carefully standardized blotting procedure. Each tissue slice of a sample was removed individually from solution and placed between two sheets of filter paper for 2 or 3 sec to remove the bulk of surface water. Each surface was then carefully wiped free of surface water using torn edges of filter paper as absorbent. At no stage of blotting was pressure applied to the tissue. The dried slices were weighed in stoppered weighing bottles. Using this procedure results were reproducible and samples could be weighed with a variation not greater than 1–2 per cent. with the tissue samples used (± 5 mg/g).

Coleoptile length was measured by transferring the sections to a well-slide on the graduated mechanical stage of a low-power microscope. Coleoptiles remained sufficiently straight for measurement when floated on solution.

III. RESULTS

(a) *Auxin-induced Plasmolysis Shift*

The results of incipient plasmolysis determinations on artichoke tissue in the presence and absence of auxin are recorded in Figure 1. In the presence of IAA plasmolysis usually occurred in more dilute solution than in untreated tissue. The auxin-induced shift in the point of incipient plasmolysis occurred within the time (*c.* 15 min) taken for the section to equilibrate with the external osmotic solution. The shift in the point of incipient plasmolysis value occurred in the presence of osmotic solution and during the lag period in auxin-induced expansion. Since there was no increase in cell volume the shift was not due to dilution of the cell sap.

Thèse experiments were repeated using naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Essentially similar results were obtained.

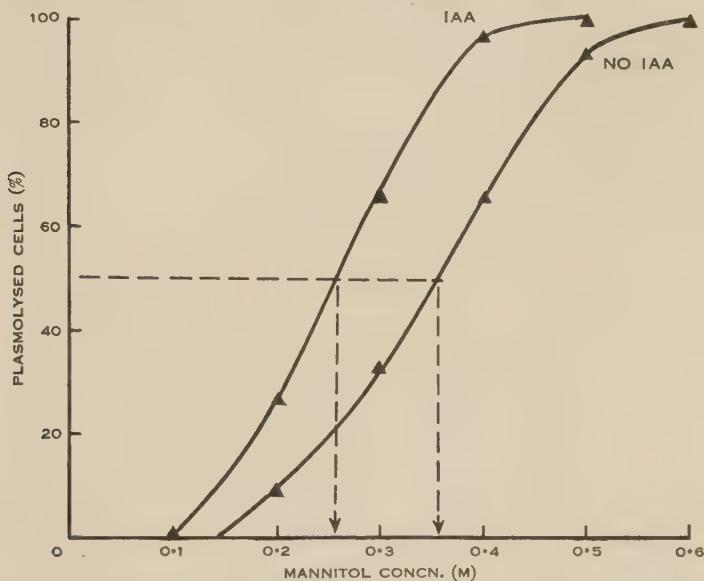


Fig. 1.—Percentage of plasmolysed cells of artichoke tissue in a range of mannitol concentrations. IAA concentration 5 mg/l. Incipient plasmolysis (50 per cent. cells plasmolysed) is indicated by arrows.

The effect of different auxin concentrations on the magnitude of the plasmolysis shift was determined. The results (Fig. 2(a)) may be compared with the auxin activity/concentration curve for auxin-induced expansion (Fig. 2(b)). Since the

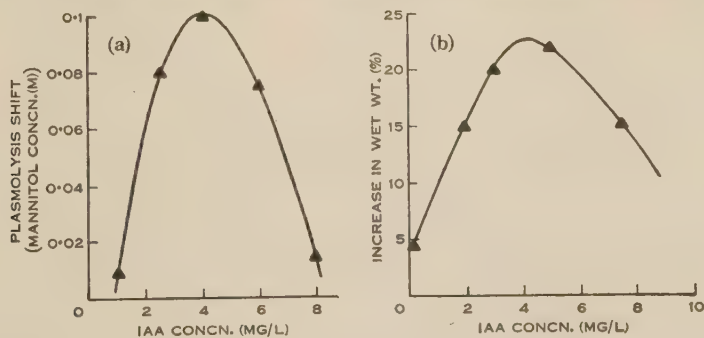


Fig. 2.—(a) Relation between auxin-induced plasmolysis shift (expressed as molar concentration of mannitol) and (b) between auxin-induced expansion and IAA concentration.

optimum concentration for water uptake caused the maximum shift in the point of incipient plasmolysis, it is suggested that the two effects, although separated in time, were closely related.

(b) *Development of a Lag Period in Storage Tissue*

The time course of auxin-induced expansion of artichoke tissue of different physiological age is recorded in Figures 3(a) and 3(b). Immature tubers harvested before dormancy showed no lag in auxin-induced expansion. This result confirms a similar observation by Hackett and Thimann (1952), which Hanson and Bonner

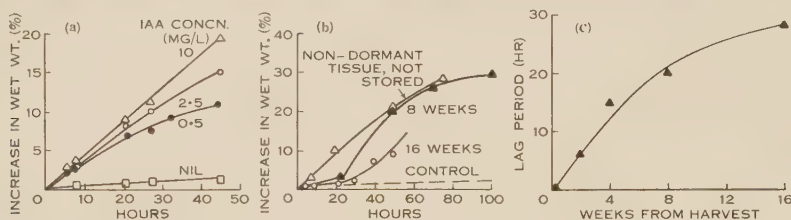


Fig. 3.—(a) Time course of expansion of immature artichoke tissue in a range of auxin concentrations. (b) Time course of auxin-induced expansion of artichoke tissue after different periods of storage at 8°C. Results are recorded for the optimum auxin concentration in each case. (c) Development of a lag period in auxin-induced expansion of artichoke tissue with storage at 8°C. The lag period associated with the optimal auxin concentration is plotted in each case.

(1955) suggested “may not be real, but a fortuitous result of the selection of two points on a sigmoid curve which happen to fall in line with the origin”. Extrapolations of the progress curves (Fig. 3(a)) pass through the origin, suggesting that an immediate increase in wet weight was obtained with a number of different IAA concentrations. Similar linear progress curves were obtained using NAA and 2, 4-D.

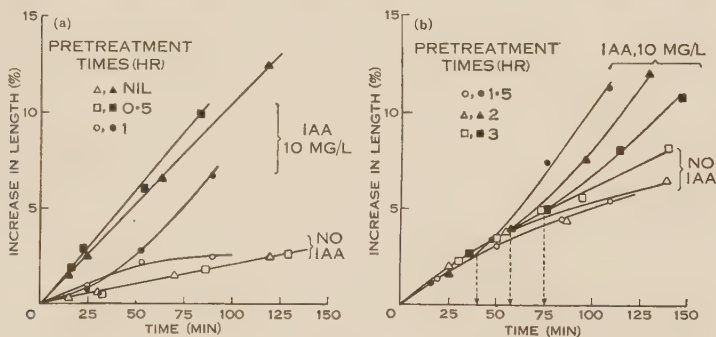


Fig. 4.—(a) Time course of wheat coleoptile elongation for sections in water and IAA (10 mg/l) after different periods of pretreatment with isotonic mannitol (0.2M). (b) Further effects of different periods of pretreatment with isotonic mannitol on the time course of wheat coleoptile elongation. Arrows indicate end of the lag period.

When immature tubers were stored or as maturity was reached in the ground, a lag period developed rapidly and increased in length, the maximum lag period being associated with fully dormant tissue (Fig. 3(c)).

(c) *Induced Lag Period in Coleoptile Elongation*

The lag period in artichokes appears to be confined to non-expanding tissue. This suggests that a lag period in coleoptiles might be induced by preventing their elongation prior to auxin treatment.

The expansion of wheat coleoptile sections was stopped for varying times by floating the sections on isotonic mannitol. The sections were then transferred to buffered auxin and the time course of elongation followed. The progress curves illustrated in Figures 4(a) and 4(b) show that a lag period in auxin-induced water uptake was observed when elongation was prevented prior to auxin treatment.

Figure 5 shows the relationship between the time of pretreatment with isotonic mannitol and the length of the induced lag period estimated from the progress curves. Initially the changes occurring in the cell were not sufficient to affect auxin-induced elongation. A resistance to expansion then developed rapidly.

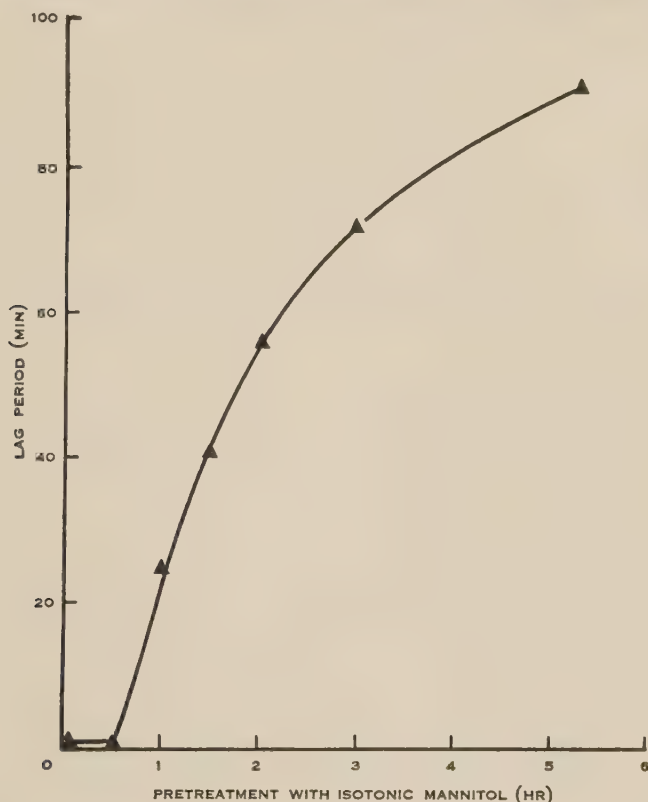


Fig. 5.—Relation between length of the induced lag period in coleoptiles and time of pretreatment with isotonic mannitol.

The initial rates of auxin-induced elongation occurring after the lag period, calculated from progress curves for each pretreatment, are recorded in Figure 6. The decreasing rate of auxin-induced expansion after increasing pretreatment indicated that the resistance of the tissue to expansion was not fully overcome during the lag period.

Different lag periods were obtained by pretreating tissues with a range of hypotonic concentrations for a constant time. The duration of the induced lag period increased with increasing concentration of the pretreating solution and was inversely proportional to the elongation occurring during pretreatment (Fig. 7).

IV. DISCUSSION

The results have shown a lag period in auxin-induced expansion of artichoke parenchyma which is related to tissue maturity; immature tissue had no lag period. A lag in auxin-induced coleoptile elongation was caused by preventing expansion prior to auxin treatment. The lag period increased with length of pretreatment in isotonic concentrations and was inversely proportional to the elongation during pretreatment in a range of hypotonic concentrations. In tissues which showed auxin-induced expansion, auxin caused a rapid plasmolysis shift which was not due to dilution of cell sap. These results can now be considered in relation to known effects of auxin on water uptake and cell expansion.

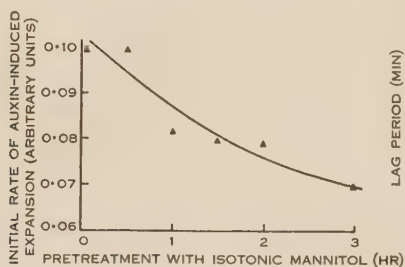


Fig. 6

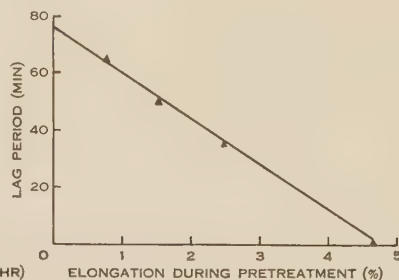


Fig. 7

Fig. 6.—Initial rate of auxin-induced coleoptile elongation after the lag period as a function of time of pretreatment with isotonic mannitol.

Fig. 7.—The lag period in auxin-induced coleoptile elongation as a function of the elongation which occurred during 3 hr pretreatment with a range of hypotonic concentrations (0.05, 0.1, 0.15, and 0.2M) mannitol.

It has been established that the auxin-induced increase in wet weight and volume of storage tissue is induced by a change in the wall pressure component of the osmotic system (McLaren 1955). Similarly, Ordin, Applewhite, and Bonner (1956) using *Avena* coleoptile sections have shown that auxin induces an osmotic uptake of water. Following Heyn's original hypothesis (see Heyn 1940) that auxin affects cell wall plasticity, reactions involving auxin and methylation of cell wall pectins have been suggested or investigated by several workers (Bennet-Clark 1955; Ordin, Cleland, and Bonner 1955; and Glasziou 1957a, 1957b).

Two possible causes of the lag period may be suggested. A lag in auxin-induced water uptake could be due to a delay in the plasticizing effect of auxin on the wall. Alternatively, the overall structural rigidity of the wall may be sufficient to prevent expansion despite a plasticizing effect of auxin on the pectic components. It is also possible that both causes may operate; for instance, the plasticizing action of auxin could be limited by the low availability of reactive sites in a structurally rigid wall system.

It is unlikely that there is an absolute delay in the plasticizing effect of auxin on the wall. Auxin induces a rapid shift in the point of incipient plasmolysis of certain storage tissues. Potato tuber tissue and the staminal hairs of *Tradescantia*, which, like artichoke tissue, show auxin-induced expansion also show a plasmolysis shift; in contrast, carrot and beet parenchyma show neither auxin-induced expansion

nor plasmolysis shift. Since this shift during the lag period is closely related to the subsequent auxin-induced expansion, it is suggested that there is no delay in the effect of auxin on the cell wall. In view of the known effects of auxin on cytoplasmic viscosity and cell wall, it is postulated that this shift reflects a reduction in cytoplasm wall adhesion which allows plasmolysis to occur in a lower external osmotic concentration.

If cell wall rigidity is the factor underlying the lag period, this rigidity must be reduced before auxin-induced expansion can occur. Wall rigidity could be reduced gradually if auxin causes a cumulative loosening of pectic components with a weakening of the cellulose-pectin wall mesh. A stage would be reached where turgor forces are sufficient to cause an irreversible plastic stretching of the wall, rigidity no longer limiting cell expansion. At such a stage with mature artichoke tissue there is evidence that expansion may be limited in rate by the auxin concentration (Adamson and Adamson, unpublished data 1957).

The sequence in development of a lag period in artichokes may be interpreted in terms of changing structural properties of the cell walls. Thus the absence of a lag period in auxin-induced expansion of immature tissue is associated with an expanding wall structure. When expansion of the tubers ceases, the continued deposition of cellulose or a change in the relative proportion of pectin and pectate would result in the stiffening of the cell wall, and consequently to the development of a lag period. One of the key reactions in the breaking of dormancy could be a reversal of wall stiffening, which would be reflected in a shortening of the lag period, as was noted by Hanson and Bonner (1955) for artichokes. The general finding of a lag period in the auxin-induced water uptake of storage tissue is due to use of dormant material.

The results with coleoptiles show that the length of the induced lag period was inversely proportional to the elongation which occurred during pretreatment. In addition, the length of the lag was related to the time of pretreatment with isotonic mannitol. These data support the hypothesis that the induced lag is caused by an increase in wall rigidity during pretreatment. The experiments of Heyn (1940) with *Avena* coleoptiles, which have been repeated by Cleland and Bonner (1956), showed that auxin acted on the wall when wall stiffening occurred in the absence of expansion. The failure of Cleland and Bonner to obtain auxin-induced expansion separated in time from auxin action in mannitol-pretreated tissue could have been due to the development of an induced lag period. These results, suggesting that auxin can act on the cell wall during the induced lag period, imply no absolute delay in the plasticizing effect.

The induced lag period in coleoptiles can be explained as an early stage in the transition from expanding to non-expanding wall structure. The prevention of elongation, even for short periods of time, may allow the setting of existing pectic wall components by cross-linkage formation and a further stiffening due to deposition of new material on the inner wall surface. In elongating coleoptiles the balance between decreasing turgor pressure and increasing wall rigidity may in part determine the cessation of cell expansion. This could occur even in the presence of concentrations of auxin optimal for elongation.

The results presented in this paper support the conclusions of Heyn (1940) on the importance of auxin-induced wall plasticity and the presence of a wall stiffening process. It is concluded that the transition from expanding to non-expanding wall structure depends on the balance between wall metabolism and turgor pressure and that the lag period in auxin-induced expansion is due to increasing structural rigidity associated with development of the mature wall.

V. ACKNOWLEDGMENTS

The work described in this paper was carried out during the tenure of a C.S.I.R.O. Studentship (Heather Adamson) and a University Studentship in Plant Physiology (D. Adamson).

The authors wish to thank Dr. R. N. Robertson, Associate Professor F. V. Mercer, and Professor R. L. Crocker for their interest and advice throughout the course of the work.

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THE EFFECT OF DEVELOPING EMBRYOS ON PLANT VIRUSES

By N. C. CROWLEY*

[*Manuscript received March 8, 1957*]

Summary

The theory that inactivation of tobacco mosaic virus by developing embryos prevents its seed transmission was tested by growing tomato embryos in media containing the virus. In many trials, using a variety of techniques, no evidence of any virus-inactivating activity was detected.

I. INTRODUCTION

Crowley (1955) demonstrated the presence of virus inhibitors in the seed of several plants, but found no virus inactivators. Hence the theory that inactivators in seeds prevent the seed transmission of plant viruses must be abandoned, unless the inactivator is some transitory product of the metabolism of embryos. The investigations described here were made to determine whether such transitory virus inactivators do occur. The relevant literature has been reviewed recently by Bennett (1956) and Crowley (1955).

Evidence will be presented in the next paper (Crowley 1957) that several of the highly infectious plant viruses commonly infect the testa, and sometimes the endosperm. Despite this the embryo develops without becoming infected. Ignoring the reasons for the lack of microspore or macrospore infection, some mechanism must exist to prevent infection of the embryo during its development. Two mechanisms have been postulated: (1) that the lack of plasmodesmatal connections prevents infection; and (2) that viruses are inactivated in the endosperm, or at the interface of the embryo and endosperm. There is general agreement among embryologists and anatomists that there are no plasmodesmatal connections to embryos, and among virologists that the likely path for movement of viruses between cells is by way of the plasmodesmata. However, as all other tissues of plants are connected by plasmodesmata it is impossible to detect experimentally any other means of between-cell movement of virus particles.

The second suggestion that viruses are inactivated in the endosperm seems a reasonable alternative. If a virus is able to multiply actively in the endosperm and if the endosperm is largely (and in many seeds totally) absorbed by the developing embryo some explanation for the lack of embryo infection is necessary. All other metabolites are absorbed by the embryo as small molecules, presumably following the breakdown of larger molecules by enzymes excreted by the embryo. It would therefore seem possible for viruses in the endosperm to be broken down similarly. This theory can be investigated even if only indirectly. If embryos do in fact prevent their own infection by breaking down viruses present in the surrounding medium, then embryos growing in a culture medium containing virus should break down at least a portion of this virus. It also follows that if such a mechanism does operate

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it must do so throughout the entire period of the development of the embryo, and that the stage of development of the embryo used should be unimportant in experimental work.

The assumption that the technique could detect such a virus-inactivating effect seems likely for two reasons: (1) the volume of culture medium used per embryo was, in many of the experiments detailed below, far less than that surrounding a naturally developing embryo, and (2) the virus concentration in the medium was, nearly always, less than that of naturally infected tissues. The embryos thus had less virus to inactivate when cultured in this way than under natural conditions.

II. MATERIALS AND METHODS

Tomato embryos were grown on White's medium containing tobacco mosaic virus. After periods of 1-12 days, the infectivity of the medium was compared with the infectivity of similar samples containing the same number of dead embryos

TABLE 1
EFFECT OF TOMATO EMBRYOS ON INFECTIVITY OF TOBACCO MOSAIC
VIRUS IN THE MEDIUM IN WHICH THEY ARE GROWING

Treatment	Mean Number of Lesions per Leaf			
	Expt. 1	Expt. 2	Expt. 3	Mean
Live embryos	53	37	76	55
Dead embryos	55	35	63	51
Without embryos	57	28	51	45

(killed by boiling for 5 min) and with similarly treated samples of medium containing no embryos. All infectivity assays were carried out in a randomized block design on *Nicotiana glutinosa*. The tobacco mosaic virus used in all experiments was diluted from a purified preparation kindly provided by Dr. R. J. Best. It was sterilized by filtration through a Lloyd's sintered-glass filter (Lloyd 1945). On several occasions the infectivity of the embryos cultured in the virus solution was tested by washing them in water, rinsing in "Teepol", again washing, and inoculating (in lots of 20 or more) to *N. glutinosa*. None was found to be infected. In this respect the cultured embryos behave as they do *in vivo*. Apparently the damage sustained by the embryos in dissection is not adequate for their infection under embryo-culture conditions.

III. RESULTS AND DISCUSSION

In the first experiments, tomato embryos were placed, 10 per tube, in 0.5 ml of a culture medium containing a 1/1000 dilution of a preparation of tobacco mosaic virus (containing 6 mg of virus protein/ml). They were incubated for 14 days at 25°C, and assayed. The results (Table 1) of three trials provided no evidence of any reduction by the growing embryos of the infectivity of the medium. In two experiments there was an insignificant increase in the infectivity of the medium. This

is attributed to the fact that the embryos used in these experiments were almost mature and in the course of their growth absorbed water from the medium, and so concentrated the virus.

(a) *Aerobic Embryo-culture*

Some workers have suggested that, under the relatively anaerobic conditions of complete immersion, embryos do not develop normally but tend to germinate immediately. This change, if it does occur, could significantly alter the metabolism of the embryos, including their effect on virus particles present in the surrounding medium. Two techniques were used to overcome this difficulty. The first was to shake the culture tubes either constantly or intermittently throughout the experiments. The results of such experiments were in every way similar to those in which the culture tubes were unshaken. The second technique was to place the embryos on

TABLE 2
EFFECT OF TOMATO EMBRYOS ON THE INFECTIVITY OF
TOBACCO MOSAIC VIRUS

Values are the mean number of lesions per half leaf
(20 replicates)

Treatment	Dilution of Virus Preparation in Medium		
	1/100	1/1000	1/10,000
Live embryos	15.6	18.8	2.4
Without embryos	17.2	13.5	1.0

strips of filter paper extending down into the medium. Using this technique the embryos developed normally, and germination in culture occurred only when mature embryos were used. The results in Table 2 are of one experiment in which this technique was used. Three dilutions of the virus in the medium were used, to enable the more certain detection of any inactivation caused by the growing embryos, for, if the virus is being inactivated, the lower the concentration of the virus the greater the proportion of the virus that must be inactivated. The results of this and several similar experiments gave no indication of virus inactivation.

(b) *Variations in the Composition of the Medium*

The physical and chemical properties of the culture medium also influence, to some extent, the metabolism of the embryos, and might affect the results of these experiments. The following variations were tried:

- (i) The sugar concentration of the medium was varied from 2 to 8 per cent. because this had been reported by Rappaport (1954) to have a pronounced effect on the development of embryos in culture.

- (ii) The medium was used at pH 4, 5, 6, 7, and 8, because acidity could greatly affect the development of the embryo, and the infectivity of the virus.
- (iii) Coconut milk, both sterile and unsterile, was added to the medium. This was found by van Overbeek, Conklin, and Blakerslee (1942) to contain excellent growth stimulators.

In none of these experiments was evidence of virus inactivation detected, nor did any of the modifications significantly effect the development of the embryo. The medium used finally contained 2 per cent. sucrose, and was adjusted to pH 7.

TABLE 3
EFFECT OF DEVELOPING TOMATO EMBRYOS ON TOBACCO MOSAIC VIRUS AT
DIFFERENT INCUBATION TEMPERATURES

Values are the mean number of lesions per half leaf

Virus Concentration in Medium	Incubation Temperature (°C)	Incubation Time		
		1 day	3 days	6 days
1/100	3	176	201	181
	25	179	138	154
1/1000	3	37	27	31
	25	35	29	21
1/10,000	3	4.7	5.2	5.0
	25	4.5	3.5	3.5

A further modification was to use embryos of different ages. Some experiments were made with the youngest embryos that could be dissected, others with half mature or mature embryos. No differences were detected and the age of the embryo is considered unimportant; any mechanism preventing embryo infection must operate throughout its development. For most of the experiments described the embryos used were between one-half and two-thirds mature, because at this stage they are growing rapidly and are easily dissected and handled.

(c) *Combined Effect of Embryo and Endosperm*

A further possibility worth investigating was that the inactivating mechanism could be of such a nature as to require the presence of the endosperm for its operation. To test this, both embryo and endosperm were cultured as a whole by using very immature tomato seeds in which the endosperm was still almost gelatinous and could be removed from the seed intact. These endosperms, together with the embryo developing within them, were then cultured in the manner described above. No evidence of virus inactivation was obtained.

The addition of dead embryos to the medium sometimes appeared to increase its infectivity. This difference could not be attributed to the absorption of water from

the medium by the embryos because the embryos, having been boiled in sterile distilled water, are far from dry when cultured. As they do not grow, they would not take up more water. Both treatments contain pieces of filter paper of the same size; thus absorption of virus particles on to the filter paper is not the cause. The most probable explanation is that some substances are lost into the medium from the embryos, which through being boiled are presumably "leaky". This could also account for the fact that the live embryos did not increase the infectivity of the medium as much as the dead embryos. It is possible that the live embryos do equally increase the infectivity of the virus, but the increase is masked by a simultaneous virus-inactivating process, with the result that neither process is detectable. Experiments were therefore carried out in which this difference between leaky (boiled) and

TABLE 4
EFFECT OF REPEATED ADDITIONS OF TOMATO EMBRYOS ON THE INFECTIVITY
OF TOBACCO MOSAIC VIRUS

Values are the number of lesions per half leaf. Differences between temperature treatments not significant at $P = 0.05$

Virus Concentration in Medium	Incubation Temperature (°C)	Incubation Time		
		1 day	2 days	4 days
1/50	3	37	45	41
	25	28	36	36
1/500	3	3.2	5.5	5.5
	25	2.1	3.0	4.7
1/5000	3	0.6	0.8	1.0
	25	0.3	0.8	0.4

"non-leaky" (live) embryos was eliminated by using only live embryos cultured at two temperatures, 3 and 25°C. The effects of a virus-inactivating mechanism should be detectable by a comparison of the infectivity of the media, because the virus inactivation, being a physiological reaction, would be accelerated by a factor of four times for a 20°C rise in temperature. The results of one such experiment are given in Table 3.

From these results it is clear that at 3°C the infectivity of the medium remained relatively constant for the duration of the experiment at all three virus concentrations. At 25°C, there appears to be a reduction in the infectivity of the virus at the 1/1000 dilution, but such a conclusion is inconsistent with the results at the lowest dilution, where the same number of embryos did not significantly reduce the infectivity of one-tenth of the amount of virus. Because of this, and the fact that at the lowest virus concentration the change in infectivity did not continue throughout the experiment, the apparent drop in infectivity is thought not to be real.

(d) *Effect of Repeated Additions of Embryos to the Medium*

One further possibility, by which the technique used could fail to demonstrate the presence of a virus-inactivating mechanism, is that such a mechanism might not continue to operate in artificial embryo-culture. Although no reason is known why this should be so, an attempt was made to obtain evidence that would eliminate even this possibility. In several experiments 10 embryos were added to each tube daily. Previously, they had been added only at the beginning of the experiment. In this way any decline in the virus-inactivating activity of the cultured embryos would be counteracted by the repeated additions of more embryos. The results of one experiment carried out in this manner are shown in Table 4.

The experimental error is high because the technique involves unavoidable sampling errors, diluting errors, and slight variations in the treatments. All the inocula in which embryos were growing at 25°C were less infective than inocula in which they had been growing at 3°C. This is attributed to the secretion, by the growing embryo, of some substances that inhibit the infectivity of the virus. It is not thought to be due to inactivation of the virus by the embryos, because the reduction in infectivity is as great, or greater, in the most concentrated inocula as in the most dilute, and the reduction in infectivity does not increase with increasing incubation time. For these reasons it is concluded that these results give no indication of any virus-inactivating activity of the developing embryos.

Some other explanation must therefore be sought to explain the means by which embryos can remain virus-free while developing within infected tissues, and, of the explanations that have been advanced, the most adequate is Bennett's (1956) suggestion that viruses are unable to infect the developing embryo because it lacks plasmodesmatal connection with other tissues.

IV. ACKNOWLEDGMENTS

I wish to thank Dr. C. G. Hansford for much help in the preparation of the manuscript, and Miss Yvonne Cuddihy for much able technical assistance.

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STUDIES ON THE SEED TRANSMISSION OF PLANT VIRUS DISEASES

By N. C. CROWLEY*

[Manuscript received March 8, 1957]

Summary

Investigations were made to explain the rarity of seed transmission of plant virus diseases. Five mechanically transmitted viruses were used, none of which induced enough sterility in their hosts to account for their lack of seed transmission.

All of the viruses studied infected the seeds of their hosts, but only the seed-transmitted bean mosaic virus was found to infect the embryos.

No evidence has been found for the presence of virus inactivators in seeds or developing embryos.

Evidence is presented that the rarity of seed transmission is due to the inability of most viruses to infect mega- or microspore mother cells of infected plants together with the inability of viruses to infect the developing embryo because of the lack of plasmodesmatal connection with the endosperm.

I. INTRODUCTION

Of several hundred plant virus diseases that have now been described, only 45 are reported to be seed transmitted. These are tabulated in Appendix 1, which shows that: (1) the transmission of plant viruses through seeds is not, as has often been claimed, more common in the Leguminosae than in some other families; (2) in only four plants does the percentage transmission exceed 50; (3) the property of transmissibility is not a property of any virus, nor of any host, but is clearly an interaction of the two, virus and host. Four hypotheses have been put forward to explain the rarity of seed transmission:

Hypothesis 1.—Allard (1915) suggested that virus infection “may disturb the normal relations of stamens and pistils to such an extent as to cause sterility”. In 1952 Caldwell demonstrated that in the aspermy disease of tomato “the presence of the virus in the microspore mother cell results in a complete interference with the normal stages of meiosis . . .”. This hypothesis adequately explains the failure of tomato aspermy virus to pass through the seed of tomato, but cannot be extended to explain why seed transmission of other viruses is so rare when infected plants produce large quantities of seed. This hypothesis also fails to explain how embryos develop to maturity in a virus-infected endosperm without themselves becoming infected.

Hypothesis 2.—Bennett (1936) suggested that the lack of vascular connection between the embryo and its parent plant prevents the seed transmission of those virus diseases which are largely limited to the vascular tissues. This theory adequately explains the absence of seed transmission of all virus diseases of this type, but again is not capable of extension to other virus diseases.

Hypothesis 3.—Duggar (1930) suggested that the seed transmission of those highly infectious viruses, which are almost ubiquitous in their distribution within a plant, might be prevented by the inactivating action of some “specific protein or

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other specific material" in the seeds. This hypothesis was elaborated by Kausche (1940), but neither of these workers distinguished between virus inactivators and virus inhibitors in seeds. Until their "inactivators" are proved not to be inhibitors their conclusions are unjustified.

Hypothesis 4.—Bennett (1936) also suggested that the lack of plasmodesmatal connection between the embryo and the parent plant prevents the seed transmission of even the most infectious virus diseases. This suggestion rests on two assumptions, and neither can be tested experimentally: (1) that the micro- and megaspore mother cells either escape infection, or are unable to support virus multiplication; (2) that the only path of intracellular virus movement is via the plasmodesmata.

The investigations described here were made to determine (i) whether virus-induced sterility could be the reasons for the rarity of seed transmission of some of the more infectious plant viruses; (ii) what tissues of the seed are infected by these viruses; (iii) whether seed-transmitted viruses infect the embryos of their hosts before or after fertilization.

II. EXPERIMENTAL AND RESULTS

(a) *The Effect of Virus Infection on Pollen and Seed Production*

An essential requirement for investigations associated with the seed transmission of plant virus diseases is a knowledge of whether viruses are able to infect microspores or macrospores, and whether this infection interferes with the normal behaviour of these cells.

Caldwell (1952) showed that with tomato aspermy disease seed transmission is made impossible by virus-induced abortion. He suggested that this may be the usual consequence of infection of microspores or macrospores by a virus, and that when virus infection does not induce abortion, seed transmission will occur. There is evidence which seems to support Caldwell's suggestion. Tobacco ringspot virus induces no sterility in soybean in which it is seed transmitted (Desjardins, Latterell, and Mitchell 1954), but does induce sterility in both petunia and tobacco where it is not seed transmitted (Henderson 1931; Valteau 1941). Similarly, a non-seed-transmitted mosaic disease of *Dolichos biflorus* was found by Uppal (1931) to reduce fertility greatly, and a graft-transmissible disease of tobacco tested by Kostoff (1933) induced complete sterility of infected plants. However, these results do not establish whether the reduced fertility of these infected plants results from the stunting and impairment of vigor induced by the virus, or from a specific effect of the virus on microspores or macrospores.

The results of Blakerslee's (1921) investigations with the Quercina disease of *Datura stramonium* seem to be at variance with Caldwell's suggestion, for Blakerslee found that, although virus infection induced a high degree of sterility, 100 per cent. of the pollen produced on infected plants was infected. In this case, at least, virus infection does not invariably lead to the abortion of pollen mother cells. Unfortunately there is no evidence to indicate whether the sterility induced by this virus results from an interference with the meiotic divisions or from a physiological disturbance of floral development.

If Caldwell's suggestion is assumed true, the rarity of reports of virus-induced sterility is surprising, particularly with highly infective viruses such as tomato spotted wilt, cucumber mosaic, or tobacco mosaic virus. Nixon (1956) estimated that the number of particles of tobacco mosaic virus per cell of tobacco leaf tissue is of the order of 6×10^7 . If their concentration in floral tissues is anything approaching this, there should be a significant reduction in fertility, unless some mechanism prevents the infection of the gametophytic tissues, or the survival of viruses in them. Allard (1915) reported a reduction in the seeds produced by mosaic-infected tobacco plants, but there are no reports of any effect of this virus on the seed production of any of its other hosts.

Few critical investigations on this aspect of the effect of plant viruses on their hosts have been done; the effects, therefore, of four viruses on the fertility of their hosts were studied in the present work. The viruses used were bean yellow mosaic, bean mosaic, tobacco mosaic, and cucumber mosaic.

(i) *Effect of Bean Mosaic and Bean Yellow Mosaic Viruses on the Fertility of Bean.*—The two viruses of bean were used because the yellow mosaic virus is not seed transmitted and the common mosaic virus is transmitted through both seed and pollen. An obvious conclusion is that if the primary cause of the lack of pollen infection by bean yellow mosaic virus is the abortion of the microspores then the virus should have a marked effect on fertility. The bean mosaic virus used was provided by Mr. J. Johnson, Queensland Department of Agriculture and Stock, Brisbane. It did not infect peas or clovers, and in beans it induced symptoms closely corresponding to those described by Reddick and Stewart (1919). The bean yellow mosaic virus used was isolated from some naturally infected bean plants at Mt. Gambier, S.A., and infected bean, pea, *Trifolium incarnatum* L. and *T. hybridum* L.: in all of these hosts the symptoms corresponded closely to those described by Pierce (1934).

Two experiments were carried out with these viruses. The first was begun early in the summer of 1955. It was repeated early in 1956, but with all plants raised in a cooled glass-house so that the symptoms of the diseases would not be masked by high temperatures. Thirty-three bean seedlings (var. Canadian Wonder) were used in the experiments, and were allocated at random to each treatment. Eleven plants were inoculated with bean mosaic virus, eleven with bean yellow mosaic virus, and eleven were left uninoculated. Throughout the growing period, records were kept of the number of flowers appearing daily on each plant, of the number of pods produced, and, after harvest, of the number of seeds per pod in each of the three treatments. The results of these experiments are given in Table 1.

The best estimate of plant fertility is the number of seeds produced per flower. On this basis a slight reduction in fertility was produced by both viruses, but it was not sufficient to explain the lack of seed transmission of bean yellow mosaic virus.

(ii) *Effect of Tobacco Mosaic and Cucumber Mosaic Viruses on the Fertility of Pungent Pepper.*—The hypothesis of virus-induced sterility could be tested more satisfactorily with viruses that reach high concentrations in their hosts. If sterility does result from the infection of the megaspores and pollen grains by viruses, then those viruses which are in highest concentration should induce most sterility. Tobacco

mosaic and cucumber mosaic viruses were chosen as suitable for this investigation. The tobacco mosaic virus was isolated from pungent pepper seed (*Capsicum frutescens* L.) obtained from Dr. N. H. McKinney, who reported 22 per cent. seed transmission to occur in this host (McKinney 1952). The cucumber mosaic virus was provided by Mr. L. L. Stubbs, Dept. of Agriculture, Burnley, Vic., and originated from naturally infected cucumbers. It infected *Cucumis sativa* L., *C. melo* L., *Cucurbita pepo* L., *Citrullis vulgaris* Shrad., *Nicotiana tabacum* L., *Lycopersicon esculentum* Mill., *Datura stramonium* L., and *Vigna sinensis* (L.) Endl. ex Hassk. and produced symptoms consistent with those described by Doolittle (1920).

TABLE 1
EFFECT OF VIRUS INFECTION ON THE FERTILITY OF BEAN PLANTS

Virus	Flowers per Plant	Pods per Plant	Seeds per Pod	Experiment 1 Seeds per Flower	Experiment 2 Seeds per Flower
None	28	10	3.8	1.4	1.1
Bean mosaic	33	10	4.1	1.3	0.4
Bean yellow mosaic	25	8	3.2	1.0	0.7

It was hoped to determine the effect of two strains of tobacco mosaic virus on the fertility of pepper, in which only one strain was seed transmitted. However, pungent peppers inoculated with the ordinary strain of tobacco mosaic virus produced only local lesions on the inoculated leaves, and systemic symptoms did not develop. It was also found that although the pepper strain of this virus is seed transmitted in pungent pepper, the embryos are not infected, but become infected by contamination from the infected testa during germination. A similar occurrence with the "tomato streak" strain of tobacco mosaic virus was described by Chamberlain (1950) in tomato.

These results thus prevented the investigation of this aspect of the problem using these two strains of a single virus on *C. frutescens*. Instead, the effect of the two viruses, tobacco mosaic and cucumber mosaic, on the fertility of pungent peppers was investigated. Experiments were carried out as described above with bean, using eight plants in each treatment.

These results (Table 2) show that tobacco mosaic virus did not affect the fertility of pungent pepper plants, whereas cucumber mosaic virus reduced their fertility by between 50 and 80 per cent. Comparisons of the pollen grains from both healthy and cucumber mosaic-infected plants did not reveal the presence of any abnormal pollen. Examinations of anthesis at an earlier stage gave no indication that either virus disturbed meiosis, and tetrads of microspores were regularly found in young anthers of infected plants. It is unlikely that a 50 per cent. reduction in the fertility of plants could be produced by interference with the normal process of meiosis without some microscopically visible effect being produced on both anthesis and pollen production. On the other hand, cucumber mosaic virus greatly upsets the normal growth of pepper plants. Infected plants are stunted, flower production is

disturbed, and they produce fewer flowers over a much longer period, and fruits are greatly distorted. It is therefore concluded that cucumber mosaic virus reduces the seed production of pungent pepper plants primarily by upsetting the normal hormonal control of plant growth with the result that fruit growth is abnormal and stunted and seed production is much reduced.

It is concluded that although virus-induced sterility may be an adequate explanation for the lack of seed transmission of tomato aspermy disease, it does not appear to be a contributing factor to the lack of seed transmission of the viruses investigated. There is no evidence that these viruses interfere with the meiotic divisions of their hosts, and, as neither is pollen transmitted, it would seem that some other mechanism must exist to prevent the infection of micro- and macrospores.

TABLE 2

EFFECT OF TOBACCO MOSAIC AND CUCUMBER MOSAIC VIRUSES ON THE FERTILITY OF PUNGENT PEPPER PLANTS

Experiment No.	Virus	Flowers per Plant	Fruits per Plant	Seeds per Fruit	Seeds per Flower
1	None	82	18.6	134	30.4
	Cucumber mosaic	82	5.1	91	5.7
	Tobacco mosaic	89	16.9	135	25.7
2	None	70	7.0	112	11.1
	Cucumber mosaic	44	2.2	43	2.2
	Tobacco mosaic	43	7.2	88	15.0

(b) *The Location of Viruses in the Seeds of their Hosts*

As the viruses studied did not induce abortion of the micro- or macrospores it was concluded that either (1) infection of these cells does not kill them; or (2) viruses are excluded from gametophytic tissues. Assuming the first to be true, embryos must be infected early in their development and seed transmission then prevented by the inactivation of virus in the embryo during seed maturation. If the second alternative is true then embryos are not infected initially, and seed transmission can still occur unless infection of the developing embryo is prevented.

Facts about the distribution of viruses in seeds are remarkably few. Different workers have reached conflicting conclusions; they have stated that in tomato seeds tobacco mosaic virus is (1) mainly a superficial contaminant (Chamberlain 1950); (2) present in the testa only (Ainsworth 1935); (3) capable of infecting testas and two-thirds of the embryos (Berkeley and Madden 1932). (The seeds used by Berkeley and Madden had already begun to germinate and may have been contaminated from the infected testa.) The distribution of only three other non-seed-transmitted viruses has been investigated. Bennett (1936) showed the curly top virus of sugar-beet to be present in all the tissues of the seed except the embryo. Sheffield (1941) obtained evidence from a study of virus inclusion bodies that "severe etch" virus infects the testa but not the endosperm or embryo of the seeds of *Hyoscyamus niger* L. Cheo

(1955) reported the infection of 100 per cent. of both embryos and testas of bean by "southern mosaic" virus. He suggested that seed transmission of this virus was prevented by the inactivation of the virus during maturation and storage of the seed. A similar suggestion was made by Gold *et al.* (1954) to explain the fact that although barley mosaic virus infected 100 per cent. of the developing embryos, only 50-90 per cent. seed transmission occurred.

The results of Ainsworth and Sheffield thus seem to support the hypothesis that viruses are unable to infect embryos and the results of Cheo and Gold *et al.* support the hypothesis that viruses do infect embryos but are inactivated before the seed germinates.

Investigations were made to determine whether embryos are infected, and whether there is any virus inactivation associated with the maturation of the seed. Five viruses were used: two viruses of bean, of which only one was seed transmitted, and three other viruses were chosen such that with each one host could be used in which seed transmission did occur, and a second host in which seed transmission had not been reported. The virus-host combinations were:

- (1) Bean mosaic virus (43 per cent. transmission (Archibald 1921)) and bean yellow mosaic virus and bean.
- (2) Tomato spotted wilt virus in cineraria (96 per cent. transmission (Jones 1944)) and tomato.
- (3) Cucumber mosaic virus in wild cucumber, *Echinocytus lobata* Mich. T. and G. (22 per cent. transmission (Doolittle and Gilbert 1919)) and cucumber.
- (4) Tobacco mosaic virus in pungent pepper (22 per cent. transmission (McKinney 1952)) and tomato.

(i) *Seed Dissection and Inoculation Techniques*.—No special techniques were necessary for the dissection of bean, cucumber, or wild cucumber seeds. The tissues were washed in water, ground in neutral composite buffer, and inoculated to test hosts. The presence of bean viruses was tested for by inoculation to five 10-14-day-old bean seedlings (var. Canadian Wonder) and for cucumber mosaic virus by inoculation to the cotyledons of five young cucumber seedlings (var. Long Green). The dissection of seeds of cineraria, tomato, and pepper was simply accomplished when the seeds were first soaked for 1 or 2 hr. In dissecting cineraria seeds, a small cut was made at the bottom of the fruit, through which the embryo was ejected by gently pressing with a flattened needle. Similarly, young seeds of tomato and pungent pepper can be separated into testa, endosperm, and embryo. *Nicotiana tabacum* was used in all inoculations to detect tomato spotted wilt virus because it was found to be more susceptible than *N. glutinosa*.

Tomato seeds from plants infected with tomato spotted wilt virus were obtained from naturally infected field crops. The cineraria seeds were obtained from both naturally infected field-grown plants and from glass-house plants infected with either a field strain or one of four pure strains of tomato spotted wilt virus, which were provided by Dr. R. J. Best, Waite Institute. All other viruses used have been described above. The seeds from tobacco mosaic-infected fruits were dissected in the same manner as the cineraria seeds, and the tissues soaked in 10 per cent. "Teepol" for

several hours. This inactivated any virus particles that were superficially contaminating the tissues, particularly those of young seeds, in which the endosperm is gelatinous and invariably contaminates the other tissues. "Teepol" was the only satisfactory surface-sterilizing agent found that inactivated superficial virus without significantly affecting the infectivity of virus contained in the tissues. Embryos dipped in a concentrated tobacco mosaic virus preparation could be freed from contaminating virus by rinsing in "Teepol", and it had a negligible effect on virus in tissues. *N. glutinosa* was inoculated to detect tobacco mosaic virus.

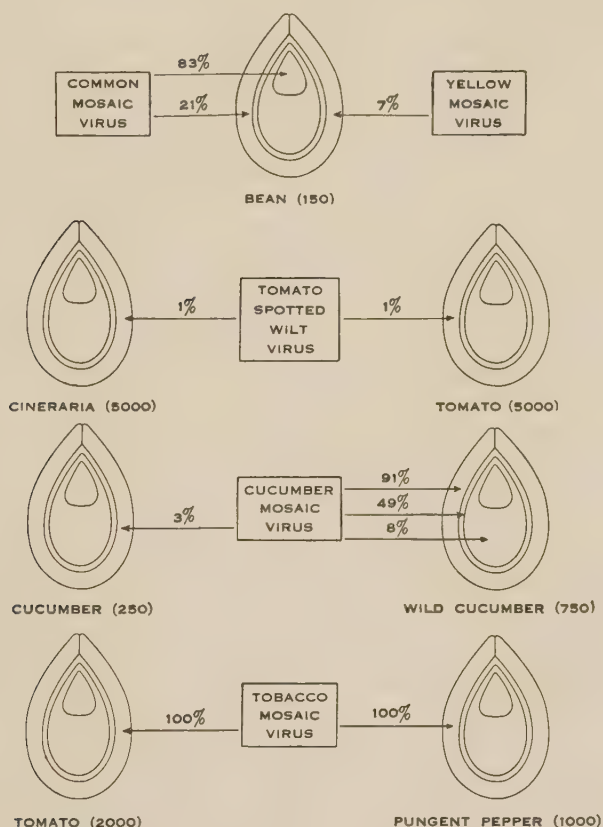


Fig. 1.—Diagrammatic representation of the distribution of viruses in the seeds of various plants. The numbers in parenthesis indicate the number of seeds dissected.

Tobacco mosaic and cucumber mosaic virus are so highly infectious that their detection by these methods presents no problem. The tissues used with the viruses of bean were large enough to ensure their detection if infected. However, tomato spotted wilt is not highly infectious and many of the tissues used were so small that the presence of the virus in the embryos of either tomato or cineraria could have escaped detection. The results of all dissection investigations are presented in Figure 1.

(ii) *Embryo-culture Experiments*.—To eliminate the possibility that the technique was not adequate to detect low concentrations of tomato spotted wilt virus, 900 tomato embryos were grown in sterile embryo-culture on solid "White's Medium" containing coconut milk (van Overbeek, Conklin, and Blakerslee 1942; White 1943). During 4–6 weeks the tomato embryos developed into small seedlings at the four- or five-leaf stage. The amount of development and active photosynthesis which had taken place by this stage would have been sufficient to allow any viruses present to multiply to easily detectable concentrations. The embryo seedlings were next ground and inoculated to tobacco. None was infected.

In all, 340 cineraria embryos were also grown in embryo-culture for 6–8 weeks and then ground and tested. Again none was infected. It was concluded that tomato spotted wilt virus does not infect the embryos of either of these hosts.

(iii) *Seed Transmission*.—It is difficult to understand how seed transmission can occur in cineraria, wild cucumber, and pungent pepper without the embryos of these seeds being infected. Several trials were carried out to determine whether seed transmission did, in fact, occur in these hosts. More than 5000 cineraria were raised from seed of plants infected with tomato spotted wilt virus. Not one infected seedling was discovered. Thus, the 96 per cent. seed transmission reported by Jones must have been obtained either with a most unusual strain of tomato spotted wilt virus, or with a different species of cineraria. A total of 400 wild cucumber seedlings were raised from the seed of plants infected with cucumber mosaic virus. Only one infected seedling was observed. Thus with this virus there seems to be no alternative to the assumption that the strain of cucumber mosaic used was not (or only very rarely) seed transmitted in the conditions of these trials. In pungent pepper, the percentage seed transmission of tobacco mosaic virus varied between 15 and 30. However, as neither embryo nor endosperm of pungent pepper is infected by this virus, seed transmission must result from the contamination of the germinating embryo from the infected testa. This was confirmed experimentally. A sample of freshly harvested pepper seeds was divided into halves: one was sown immediately and the other after the testa had been removed. 25 per cent. of the seedlings of the first lot were found to be infected when inoculated to *N. glutinosa* but not one infected seedling was detected amongst the dissected (testa removed) seed.

(iv) *Changes during Maturation*.—It has been shown above that neither tobacco mosaic nor tomato spotted wilt virus infects the embryos of their hosts at any stage, and no change in the proportion of testas infected by either of these viruses could be detected as the seeds matured. With these two viruses, therefore, the results illustrate virus distribution in seeds at all stages of development. However, the distribution of cucumber mosaic virus in the seeds of wild cucumber altered significantly during their maturation. Table 3 presents the results of one trial in which a total of 235 seeds were dissected at all stages of maturity. To calculate these results, seeds were classed as immature in which the embryo was less than half its mature size. Those in which the embryo had attained its full size and the testa had coloured were classed as mature. As the testa achieves its full size very early in seed development this arbitrary classification was easily made.

As no very young embryos and only one mature embryo was infected, a mechanism in the developing embryo must prevent its infection by viruses present in the endosperm or perisperm. There is no endosperm in mature seeds as this tissue is absorbed by the developing embryo. The decline in the percentage of testas infected could be caused by the inactivation of the virus or the production of inhibitors in the maturing testa.

The results obtained with all five viruses show that the viruses that are not seed transmitted are unable to infect the developing embryos of their hosts, and indicate that seed transmission depends on the ability of a virus to infect the embryo, even if, as with pungent pepper seeds, embryo infection occurs during germination.

TABLE 3
DISTRIBUTION OF CUCUMBER MOSAIC VIRUS IN THE SEED OF WILD CUCUMBER

	Percentage Infected:			
	Testa	Perisperm	Endosperm	Embryo
Immature	91	49	8	0
Mature	27	0	—	0.7

(c) *Effect of Environmental Factors on Seed Transmission*

All of the evidence given above strongly suggests that the lack of seed transmission of the highly infectious plant viruses is not explicable in terms of the three hypotheses that have been advanced. The viruses studied do not induce sufficient sterility to account for their lack of seed transmission: the seeds of the hosts used do not contain any virus-inactivating substances (Crowley 1955), and the developing embryos do not inactivate virus particles in the medium surrounding them (Crowley 1957). The remaining hypothesis that viruses are not seed transmitted because they are unable to infect or survive in the gametophytic cells of their hosts and are unable to infect developing embryos because of the lack of plasmodesmatal connections between embryo and endosperm cannot be investigated directly. However, it is possible to investigate a conclusion that can be drawn from this hypothesis. Those viruses which are pollen transmitted must be able to infect gametophytic cells. Most probably all viruses which are seed transmitted must also be able to infect gametes, for otherwise they must infect the developing embryo despite the lack of plasmodesmata. If the developing embryo becomes infected, the percentage of infected embryos must increase during embryo development. This has been studied with bean mosaic virus, and disproved. It was found impossible to test very young embryos and a more satisfactory method was sought.

The percentage seed transmission of bean mosaic virus varies from 20 to 60 per cent. (Harrison 1935). Environmental factors might be responsible for this variation, because of its great effect on virus concentration in plants, and through this on the

percentage of infected gametes. Temperature is probably the most important environmental factor affecting the virus concentration of bean mosaic virus in beans (Fajardo 1930), and as it is one of the most easily controlled factors, it should be possible to determine whether the percentage seed transmission of bean mosaic virus is affected by the temperature before or after fertilization. Results thus obtained should indicate when embryos become infected: before fertilization (by way of the gametes), or after fertilization (during embryo development), or both.

An experiment to obtain this information was made in two glass-houses: one was insulated by double glass walls and refrigerated to a temperature usually below 62°F, never above 75°F, and the other was equipped with thermostatically controlled heaters to keep the minimum temperature above 68°F; day temperatures here varied between 80 and 90°F.

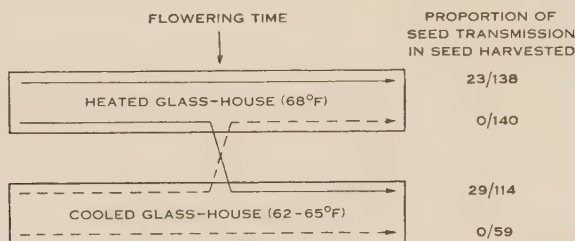


Fig. 2.—Effect of prior- and post-fertilization temperatures on the seed transmission of bean mosaic virus.

Beans (var. Canadian Wonder) were used and were inoculated with common bean mosaic virus at the primary leaf stage. A week after inoculation the plants were divided at random into four lots of 30. These groups of plants were then moved between the heated and cooled glass-houses so that an estimate could be obtained of the relative effect on seed transmission of the environment before and after fertilization. The treatments and the results are set out diagrammatically in Figure 2.

In the conditions of this experiment, only the temperature before fertilization was important in determining the percentage of seed infected with bean mosaic virus, which is consistent with the explanation that even this seed-transmitted virus is unable to infect the developing embryo. With bean mosaic virus, and possibly with other seed transmitted viruses, seed transmission depends on the ability of the virus to infect the microspores, macrospores, or embryo sac before fertilization.

III. DISCUSSION

The rarity of seed transmission of plant viruses cannot be attributed to one cause. Seed transmission is impossible for the many viruses that fall into one of the following four classes:

- (1) Those which kill their hosts.
- (2) Those which prevent flower formation.
- (3) Those which are limited in their distribution within their host.
- (4) Those which are unable to tolerate the changes that take place in the seed during its maturation and desiccation, e.g. southern bean mosaic virus.

However, these four classes include less than 10 per cent. of the known plant viruses and there must be yet other reasons for the lack of seed transmission of the remaining 90 per cent. Several theories have been put forward and each of these is now examined in relation to the evidence obtained.

Crowley (1955) demonstrated the presence of virus inhibitors in the seeds of several hosts, but failed to detect the presence of virus inactivators. Crowley (1957) also failed to detect the presence of virus inactivators in developing embryos. Duggar's (1930) theory that seed transmission is prevented by the action of virus inactivators in seeds is unsupported by evidence.

Caldwell's (1952) theory that virus-induced sterility prevents the seed transmission of plant viruses is an adequate explanation, but applies to only one or two viruses.

The only explanation for the rarity of seed transmission not at variance with the available evidence is Bennett's (1936) theory that most viruses are unable to survive in the micro- or macrospores or embryo sacs, and apparently all viruses are unable to infect developing embryos because of their lack of plasmodesmatal connections with the surrounding tissues. The results presented here show that none of the four non-seed-transmitted viruses used was able to infect the embryos of its host, and they strongly indicate that the seed-transmitted bean mosaic virus is able to infect the embryos only prior to fertilization. This theory aligns the prevention of seed transmission with the more basic problem of resistance of plants (or in this case of individual cells) to viruses, and with the genetic control of resistance. Couch (1955) found that the genotype of the host is the major factor determining the percentage seed transmission of lettuce mosaic virus, and Cation's experiments (1952) illustrate the complex nature of the interaction and the importance of the strain of the virus. He reported that in seed harvested from a single cherry tree infected with two seed-transmitted viruses, the percentage transmission of one of them (cherry ringspot) was four times as great as the percentage transmission of the other (cherry yellows). If seed transmission depends on the ability of a virus to survive in haploid gametophytic cells, it is to be expected that the genotype of both host and virus would be of major importance, and that different results would be obtained with different strains of a virus or different varieties of a host. Lindstrom (1941) reported a haploid line of tomato to be susceptible to an aucuba strain of tobacco mosaic virus, although only very slight symptoms were produced. Later, however, he reported "the haploid seems to have developed an immunity to the natural infection which was constantly affecting other tomato varieties in the same glass-house." It appears that Lindstrom's lines of haploid tomatoes did possess, if not an immunity, at least a very high degree of resistance to infection by tobacco mosaic virus, and even when infected, they did not support virus multiplication as readily as the diploid. If this resistance to virus infection is a normal characteristic of haploid cells the rarity of pollen and seed transmission is only to be expected. One test of this conclusion would be to determine whether a virus would be seed transmitted in an autotetraploid host. The gametes of such a plant would be diploid, and therefore presumably susceptible.

The low percentage of seed transmission in infected plants where it does occur is difficult to explain. The only reason that can be suggested is that viruses are not present in the nucellar and anther tissues in sufficient concentration to achieve 100 per cent. infection. The rarity of seed transmission of plant viruses has usually been regarded as a remarkable phenomenon. However, natural selection would tend to eliminate any line of plants in which the seed transmission of a severe virus disease was common. In order to be seed transmitted a virus must be able to invade its host systemically to infect and survive in the haploid gametophytic cells, and to survive in the embryo throughout its development, maturation, storage, and germination.

IV. ACKNOWLEDGMENTS

I wish to thank Dr. C. G. Hansford for help in the preparation of the manuscript, Dr. N. T. Flentje for much assistance throughout the work, and Miss Jan Martin for much able technical assistance.

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APPENDIX I
SEED-TRANSMITTED VIRUSES

Host	Virus*	Per Cent. Trans- mission	Reference
CANNABINACEAE			
<i>Humulus lupulus</i>	Chlorotic disease of hop	27	Salmon and Ware (1935)
CHENOPODIACEAE			
<i>Beta vulgaris</i>	Beet yellows	30	Clinch and Loughnane (1948)
COMPOSITEAE			
<i>Cineraria</i> sp.	Tomato spotted wilt†	96	Jones (1944)
<i>Helianthus annuus</i>	Unnamed	17-43	Traversi (1949)
<i>Lactuca sativa</i>	Lettuce mosaic	6	Ainsworth and Ogilvie (1939)
<i>L. sativa</i>	Lettuce mosaic	5	Newall (1923)
<i>L. sativa</i>	Lettuce mosaic	10	Ogilvie <i>et al.</i> (1934)
<i>L. sativa</i>	Mosaic	8	Grogan and Bardin (1950)
<i>L. sativa</i>	Yellow mosaic	30	Vasdeuva <i>et al.</i> (1948)
<i>Senecio vulgaris</i>	Lettuce mosaic	0.5	Ainsworth and Ogilvie (1939)
CONVOLVULACEAE			
<i>Cuscuta campestris</i>	Dodder latent mosaic	4.8	Bennett (1944)
CUCURBITACEAE			
<i>Cucumis melo</i>	Musk melon mosaic	28-94	Rader <i>et al.</i> (1947)
<i>C. melo</i>	Cucumber mosaic	2	Hendrick (1934)
<i>C. melo</i>	Cucumber mosaic	16	Mahoney (1935)
<i>C. pepo</i>	Musk melon mosaic	—	Rader <i>et al.</i> (1947)
<i>C. sativa</i>	Cucumber mosaic	1.4	McClintock (1916)
<i>Cucurbita pepo</i>	Squash mosaic	0.96	Middleton (1944)
<i>Cucurbita pepo</i>	Cucumber mosaic	1.5	Chamberlain (1939)
<i>Cucumis moschata</i>	Musk melon mosaic	28-94	Rader <i>et al.</i> (1947)
<i>Echinocystus lobata</i>	Cucumber mosaic†	22	Doolittle and Gilbert (1919)
GRAMINEAE			
<i>Hordeum vulgare</i>	False stripe‡	50-100	Gold <i>et al.</i> (1954)
<i>H. vulgare</i>	False stripe	86	Hagborg (1954)
<i>Triticum vulgare</i>	False stripe	71	Hagborg (1954)
<i>H. vulgare</i>	False stripe	58	McKinney (1951)
LEGUMINOSEAE			
<i>Dolichos biflorus</i>	<i>D. biflorus</i> mosaic	25-40	Uppal (1931)
<i>Glycine soja</i>	Soybean mosaic	10-25	Kendrick and Gardner (1921)
<i>G. soja</i>	Tobacco ring spot	54-78	Desjardins <i>et al.</i> (1954)
<i>G. soja</i>	Tomato ring spot	76	Kahn (1956)
<i>Lathyrus odoratus</i>	Pea mosaic	—	Dickson (1922)
<i>Phaseolus limensis</i>	Lima bean mosaic	25	McClintock (1917)
<i>P. vulgaris</i>	Bean mosaic‡	—	Reddick and Stewart (1919)
<i>P. vulgaris</i>	Bean mosaic	43	Archibald (1921)
<i>P. vulgaris</i>	Bean mosaic	20-59	Harrison (1935)
<i>P. vulgaris</i>	Bean mosaic	10-30	Fajardo (1930)
<i>P. vulgaris</i>	Red node	27	Thomas and Graham (1951)
<i>Pisum sativum</i>	Pea mosaic	0.5	Dickson (1922)
<i>Trifolium hybridum</i>	Pea mosaic	0.5	Dickson and McRostie (1922)
<i>T. pratense</i>	Pea mosaic	47	Dickson and McRostie (1922)
<i>Vicia faba</i>	Mosaic	1	Quantz (1953)
<i>Vigna sesquipedalis</i>	Asparagus bean mosaic	37	Snyder (1942)

APPENDIX I (*Continued*)

Host	Virus*	Per Cent. Transmission	Reference
LEGUMINOSEAE (<i>continued</i>)			
<i>V. sineusis</i>	Cowpea mosaic	14	Gardner (1927)
<i>V. sineusis</i>	Cowpea mosaic	7	McLean (1941)
<i>V. sineusis</i>	Cowpea mosaic	11	Yu (1946)
RUTACEAE			
<i>Citrus aurantifolia</i>	Xyloporosis	66	Childs (1956)
MALVACEAE			
<i>Abutilon</i> sp.	<i>Abutilon</i> mosaic	< 1	Keur (1933)
ROSACEAE			
<i>Prunus avium</i> (var. Mazzard)	Cherry ringspot	5	Cochran (1946)
<i>P. avium</i> (var. Mazzard)	Cherry ringspot	56	Cation (1952)
<i>P. cerasus</i>	Cherry ringspot	30	Cation (1949)
<i>P. mahaleb</i>	Cherry yellows	7.8	Cation (1949)
<i>P. mahaleb</i>	Cherry yellows	41	Cation (1952)
<i>P. mahaleb</i>	Cherry ringspot	10	Cation (1952)
SOLANACEAE			
<i>Capsicum frutescens</i>	Tobacco mosaic†	22	McKinney (1952)
<i>C. annuum</i>	Mosaic‡	—	Ikeno (1930)
<i>Datura stramonium</i>	Q disease‡	100	Blakerslee (1921)
<i>Lycopersicon esculentum</i>	Tomato streak	66	Berkeley and Madden (1932)
<i>L. esculentum</i>	Tobacco mosaic	—	Berkeley and Madden (1932)
<i>L. esculentum</i>	Tobacco mosaic	2	Doolittle and Beecher (1937)
<i>L. esculentum</i>	Cucumber mosaic	0.2	van Koot (1949)
<i>Nicotiana tabacum</i>	Tobacco ringspot	17	Valleau (1941)
<i>Petunia</i> sp.	Tobacco ringspot	20	Henderson (1931)
<i>Physalis peruviana</i>	Tomato bunchy top	29	McClean (1948)
<i>Solanum tuberosum</i>	Virus Y	16	Sprav (1951)
<i>S. tuberosum</i>	Virus Y	14	Reddick (1936)
<i>S. uncanum</i>	Tomato bunchy top	53	McClean (1948)
URTICACEAE			
<i>Ulmus campestris</i>	Elm mosaic	3	Bretz (1950)

*Names of viruses used throughout are taken from the "Virus Index" in *Rev. Appl. Mycol.*

24 (13): 515-56, 1945.

†Results not confirmed in the present paper.

‡Also recorded to be pollen transmitted.

THE METABOLISM OF *ASCARIS LUMBRICOIDES* OVARIES

III. THE SYNTHESIS OF ALANINE FROM PYRUVATE AND AMMONIA*

By J. K. POLLAK†

[Manuscript received May 22, 1957]

Summary

Small amounts of α -amino nitrogen are formed when dialysed ovary homogenates are incubated with ammonium chloride, sodium pyruvate, and various cofactors.

Under anaerobic conditions chloramphenicol and to a lesser degree ascorbic acid and large amounts of lactate enhance the reductive amination of pyruvate. The reaction is dependent on the presence of reduced diphosphopyridine nucleotide and adenosine triphosphate. Hydrogen acceptors such as methylene blue considerably decrease alanine formation.

In the presence of chloramphenicol and the necessary cofactors the optimum pH for the formation of alanine from pyruvate and ammonium chloride is 7.2; this is in contrast to the two pH optima (6.9 and 8.2) obtained for this system in the absence of chloramphenicol. A progress curve of the amination reaction was constructed.

The significance of the reductive amination of pyruvate in the formation of amino acids in the ovaries of *Ascaris lumbricoides* is considered.

I. INTRODUCTION

Ascaris lumbricoides is a parasitic roundworm commonly found in pigs. A production of over 200,000 eggs per day by female worms has been recorded (Brown and Cort 1927) and hence the ovaries of this worm provide good material for the study of amino acid and protein metabolism.

Earlier work (Pollak and Fairbairn 1955*a*, 1955*b*) demonstrated a relatively high alanine content in the non-protein fraction of the ovaries, low glutamic acid dehydrogenase activity, and a very high alanine-glutamic acid transaminase activity. A non-specific alanine- α -keto acid transaminase was also detected, while the corresponding glutamic acid- α -keto acid transaminase could not be demonstrated (Pollak, unpublished data). These facts, together with the evidence of α -amino nitrogen formation by ovary homogenates in the presence of pyruvate and ammonium chloride (Pollak and Fairbairn 1955*b*), indicate that alanine rather than glutamic acid plays the key role in amino acid metabolism. This concept is further strengthened by the fact that *A. lumbricoides* is a facultative anaerobe with a high rate of aerobic and anaerobic glycolysis and thus pyruvate would be readily available, and in addition large amounts of ammonia are present in the ovary tissue (Pollak and Fairbairn 1955*a*, Pollak 1957).

In a previous study of the amino acid metabolism of ovary homogenates of *A. lumbricoides* (Pollak and Fairbairn 1955*b*) it was demonstrated that although

*Presented in a condensed form at the 1st Annual Meeting of the Australian Biochemical Society, Sydney, 1956.

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reductive amination of pyruvate occurred in this tissue, only small yields of alanine were obtained, and in several experiments the ovary preparations were altogether inactive for this reaction. However, since the reductive amination of pyruvate may well play a central part in the amino acid metabolism of the ovaries of *A. lumbricoides*, the system has now been studied in greater detail. The present investigation concerns factors which enhance the synthesis of alanine from pyruvate and ammonium chloride.

II. MATERIALS AND METHODS

The ovaries were isolated from female *A. lumbricoides* which had been collected in 1 per cent. saline at 37°C, at Homebush State Abattoirs, Sydney. The maintenance of the worms *in vitro* and the dissection of the ovaries were described by Pollak and Fairbairn (1955a).

Between 1 and 2 g of tissue were homogenized in 6 ml of a cold 0.5 per cent. KCl solution containing 12.2 mg nicotinamide per ml. To the homogenate an equal volume of M/10 Sorensen phosphate buffer (pH 6.9 or 7.2) was added and the suspension dialysed with stirring in the cold (2°C) against 2 l. distilled water for 6–8 hr.

All experiments were carried out under anaerobic conditions in evacuated Thunberg tubes. Essentially the same results were obtained when the tubes were alternatively evacuated and gassed three times with a mixture of 95 per cent. nitrogen and 5 per cent. carbon dioxide. The tubes were incubated at 37°C. After incubation the reaction mixture (3 ml) was deproteinized by the addition of 7 ml of ice-cold ethanol. The protein precipitate was separated by centrifugation and the alcoholic extract made up to 10 ml; 0.3-ml samples were withdrawn for the measurement of total α -amino nitrogen and 5 ml was used for qualitative and quantitative amino acid chromatography as previously described (Pollak and Fairbairn 1955a, 1955b). α -Amino nitrogen was determined by measuring the optical density of the ninhydrin compound at 570 $m\mu$ in a Beckman model DU spectrophotometer. In all experiments alanine made up by far the greatest proportion of the synthesized α -amino nitrogen, therefore alanine was used to prepare the standard curve for the photometric determinations. For the measurement of total α -amino nitrogen special precautions were necessary to ensure complete removal of ammonia from the solution, as this compound also reacts with the ninhydrin reagent and thus interferes with the determination. After the addition of 0.1 ml of 0.1N NaOH, each 0.3-ml sample was left in a vacuum dessicator overnight over conc. H_2SO_4 , at a pressure of less than 14 mm Hg. Controls included with each experiment ensured that all the ammonia was removed. Another precaution had to be taken whenever chloramphenicol was present in the reaction mixture. Chloramphenicol itself reacts with the ninhydrin reagent, resulting in a high optical density at 570 $m\mu$. In this case blanks (reaction mixtures at 0 min) containing chloramphenicol with and without other components were run with every experiment to allow for the optical density attained by chloramphenicol in the absence and presence of substrates.

All substrates were obtained from commercial sources, neutralized whenever necessary, and used in concentrations as indicated in the text or in the legends of the tables and figures.

III. RESULTS

 α -Amino Nitrogen Formation

In the early experiments of this investigation it was confirmed that ovary homogenates are able to synthesize α -amino nitrogen in the presence of large concentrations of pyruvate and ammonium ions. The yield of α -amino nitrogen formed

TABLE 1

α -AMINO NITROGEN FORMATION BY OVARY HOMOGENATES OF ASCARIS LUMBRICOIDES

Each tube contained 2 ml of a 10 per cent. ovary homogenate (pH 6.9); after the addition of all the components the volume was made up to 3.0 ml with 0.1M phosphate buffer (pH 6.9). Temperature, 37°C; gas phase, vacuum; duration of experiment, 3 hr. ATP, adenosine triphosphate, DPNH, reduced diphosphopyridine nucleotide

Age of Worms	Additions	Quantity (μ moles)	Number of Experiments	α -Amino Nitrogen (μ moles/g tissue/hr)
Fresh	Ammonium chloride Sodium pyruvate	120 120	5	2.2*
Fresh	Ammonium chloride Sodium pyruvate ATP	120 120 4	5	2.1*
Fresh	Ammonium chloride Sodium pyruvate	80 80	2	0.7
Fresh	Ammonium chloride Sodium pyruvate	40 40	2	None
3 days	Ammonium chloride Sodium pyruvate	120 120	4	7.0†
3 days	Ammonium chloride Sodium pyruvate ATP	120 120 4	4	6.4†
3 days	Ammonium chloride Sodium pyruvate DPNH	120 120 1	4	6.8†
3 days	Ammonium chloride Sodium pyruvate ATP DPNH	120 120 4 1	2	6.9†

* In three other experiments no amination took place.

† In one other experiment no amination took place.

was always small and not significantly affected by the addition of adenosine triphosphate (ATP) and reduced diphosphopyridine nucleotide (DPNH) (Table 1). These

experiments demonstrated only small α -amino nitrogen production, but several consistent features were revealed. Amination occurred only when the substrates (pyruvate and ammonium ions) were present in large quantities. Ovary homogenates which were prepared from worms that had been kept up to three days in an inorganic medium (Baldwin and Moyle 1947) gave rise to greater amounts of α -amino nitrogen than did homogenates prepared from fresh worms (Table 1). A pH activity curve in the presence of phosphate buffer showed that the reaction exhibited two pH optima (Fig. 1).

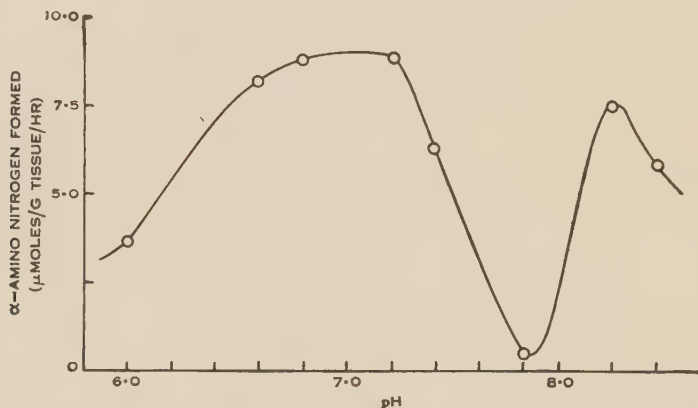


Fig. 1.—The effect of pH on α -amino nitrogen formation in ovary homogenates. Reaction mixture: 1.5 ml homogenate in 0.5 per cent. potassium chloride containing nicotinamide, 120 μ moles ammonium chloride, 120 μ moles sodium pyruvate, and 1 ml 0.1M phosphate buffer of required pH to bring the volume to 3.0 ml. Other conditions as cited in the text.

(b) *Enhancement of Alanine Formation*

In an attempt to enhance the rate of pyruvate reduction to form alanine, chloramphenicol and ascorbic acid were added to the reaction mixture. Both compounds have been used previously for the selective channelling of particular pathways of pyruvate reduction (Groth and LePage 1954). Table 2 demonstrates the effect of chloramphenicol and ascorbic acid on the rate of alanine formation from pyruvate and ammonium ions.

Chloramphenicol produced a three-fold increase, while ascorbic acid caused only a small increase in alanine formation. Chloramphenicol and ascorbic acid together caused the production of a greater amount of alanine than the addition of either one of the compounds; however, no true additive effect was obtained. Furthermore, in the presence of chloramphenicol, alanine accounted for practically all the newly formed α -amino nitrogen, with small increases in the glutamic acid concentration, while in the absence of chloramphenicol, alanine as well as aspartic acid was formed (Pollak and Fairbairn 1955b).

In the absence of chloramphenicol the addition of ATP and DPNH had no effect on the amination reaction (Table 1) but in the presence of chloramphenicol considerable increases in alanine formation were observed (Table 2). When DPNH was replaced by a hydrogen acceptor such as methylene blue, alanine formation

was greatly decreased (Table 2), indicating the dependence of reductive amination of pyruvate on a hydrogen donor such as reduced coenzyme and therefore upon anaerobic conditions.

TABLE 2
EFFECT OF CHLORAMPHENICOL AND ASCORBIC ACID ON
ALANINE FORMATION

Each tube contained 1.5 ml of a 10 per cent. ovary homogenate (pH 7.2), 120 μ moles ammonium chloride, 120 μ moles sodium pyruvate, and additions as indicated in the table. After the addition of all the components the volume was adjusted to 3.0 ml with 0.5 per cent. potassium chloride. Temperature, 37°C; gas phase, vacuum; duration of experiment, 1 hr. ATP, adenosine triphosphate; DPNH, reduced diphosphopyridine nucleotide

Additions	Quantity (μ moles)	Alanine Formed* (μ moles/g tissue/hr)
None	—	8.0
Chloramphenicol	15	15.3
Ascorbic acid	30	13.8
Chloramphenicol Ascorbic acid	15 30	17.3
Chloramphenicol Ascorbic acid ATP	15 30 4	62.0
Chloramphenicol Ascorbic acid ATP DPNH	15 30 4 1	106.2
Chloramphenicol Ascorbic acid ATP Methylene blue	15 30 4 1	17.3

*Mean values of three experiments.

In the absence of either pyruvate or ammonium ions or in the absence of both these compounds, chloramphenicol did not give rise to alanine, or any other form of α -amino nitrogen; this was confirmed in every instance by means of paper chromatography.

Chloramphenicol and ascorbic acid are thought to act by shifting the balance of the electron transport system of pyruvate reduction, so that one particular hydrogen

acceptor is favoured (Groth and LePage 1954). Therefore the addition of an end-product of pyruvate reduction other than alanine should have a similar effect, as the equilibrium of that particular reaction would be changed in such a way as to favour other pathways of pyruvate reduction. This is shown in Table 3, where the α -amino nitrogen produced by the addition of a large amount of lithium lactate is compared with the amount of α -amino nitrogen produced in the presence of ascorbic acid. The results indicate that the end-product of pyruvate reduction to lactate, and ascorbic acid which may act as an inhibitor of this reaction (Groth and LePage 1954), have the same enhancing effect on alanine formation.

TABLE 3
EFFECT OF LACTATE AND ASCORBIC ACID ON α -AMINO
NITROGEN FORMATION

Each tube contained 1.5 ml of a 10 per cent. ovary homogenate (pH 6.9), 120 μ moles ammonium chloride, and additions as shown in the table. The volume was made up to 3.0 ml with 0.1M phosphate buffer (pH 6.9). Temperature 37°C; gas phase, vacuum; duration of experiment, 1 hr

Additions	Quantity (μ moles)	α -Amino Nitrogen (μ moles/g tissue/hr)
Sodium pyruvate	120	7.0
Sodium pyruvate Ascorbic acid	120 30	13.0
Sodium pyruvate Lithium lactate	120 120	12.1
Lithium lactate	120	0.5

(c) *Effect of pH*

In the presence of chloramphenicol and other cofactors the pH curve gave a single sharp peak with an optimal pH at 7.2 (Fig. 2). This is in contrast to the pH activity curve obtained under conditions cited in Figure 1.

(d) *Time Curve of Alanine Formation*

The time curve of the reaction was followed in a dialysed homogenate with the necessary additions for optimal activity. So far all attempts to purify the enzyme responsible for the reductive amination of pyruvate have failed. Therefore no kinetic studies or rate measurements have been carried out apart from the simple progress curve of alanine formation by a dialysed homogenate (Fig. 3).

IV. DISCUSSION

The mechanism of amino acid formation from α -keto acids has been under discussion for some considerable time. The earliest experiments stem from the school led by von Euler, who showed that in microorganisms the reverse reaction

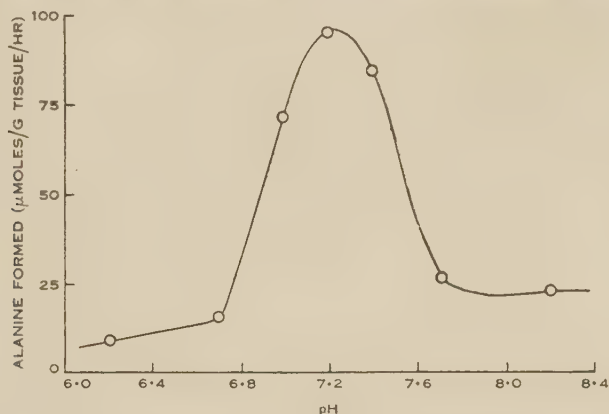


Fig. 2.—The effect of pH on alanine formation in ovary homogenates in the presence of chloramphenicol and other cofactors. Reaction mixture: 1 ml homogenate in 0.5 per cent. potassium chloride containing nicotinamide, 120 μ moles ammonium chloride, 120 μ moles sodium pyruvate, 15 μ moles chloramphenicol, 30 μ moles ascorbic acid, 4 μ moles ATP, 1 μ mole DPNH, and 0.6 ml of 0.1M phosphate buffer of required pH to bring volume to 3.0 ml. Other conditions as cited in the text.

of glutamic dehydrogenase enabled them to utilize ammonium ions for α -amino nitrogen synthesis (Adler *et al.* 1938). In 1939, Krebs and Cohen showed that a

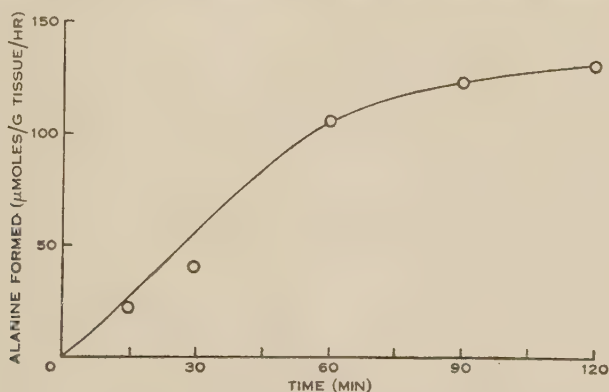


Fig. 3.—Progress curve of the enhanced alanine formation from pyruvate and ammonium chloride. Reaction mixture: optimal conditions used as shown in Table 2.

similar reaction accounted for amination of α -ketoglutaric acid in animal tissues. For many years this reaction was considered as the only pathway of α -amino nitrogen formation. In 1947, Kritzman indicated that pyruvate and oxalacetate were also

involved in amination reactions. Further experiments with animal tissues (Wiss 1948; Canzanelli, Rapport, and Guild 1950; Daugherty 1954) indicated that pyruvate might be involved in direct amination. However, contradictory results concerning the cofactor requirement of the enzyme involved and the aerobic or anaerobic nature of the reaction made the interpretation of these results difficult.

It has now been established that alanine can serve as an amino group donor in many transamination reactions (Rowell 1951; Rudman and Meister 1953), this function was formerly ascribed only to glutamic acid and aspartic acid. Hence the reduction of pyruvate to form alanine may now be regarded as an important avenue of amino acid synthesis. Furthermore in organisms in which there is a considerable excess of glycolysis over aerobic respiration, the accumulating three-carbon compound will be of greater importance as an amino group acceptor than α -ketoglutarate.

The results of the preliminary experiments (Table 1, Fig. 1), particularly the pH activity curve which exhibited two pH optima, indicated either a multiple step reaction or a competition between two or more enzymes for the same substrate (pyruvate) causing the trough in the pH activity curve. Of great interest, also, was the observation that homogenates prepared from worms which had been kept in the inorganic non-nutrient medium for 3 days were more active than homogenates prepared from fresh ovary tissue. The ammonium ion concentration of ovary tissue is known to decrease on aging the worms in such a non-nutrient medium (Pollak 1957). The enhancement of alanine formation in homogenates of aged worms could be explained either by the formation of activating substances for alanine formation or by the production of depressors of competing reactions. In the latter instance, such a compound can act as a metabolic regulator, accelerating alanine formation from pyruvate at the expense of other reduction reactions of pyruvate.

In a recent study on the metabolism of pyruvate of rat liver and tumour homogenates, Groth and LePage (1954) showed that ascorbic acid, glutathione, and chloramphenicol act as such metabolic regulators determining the reductive pathway of pyruvate. In the present investigation glutathione was considered to be unsuitable, since quantitative paper chromatography was used for the determination of the amino acids. But ascorbic acid, which in rat tissue had a similar effect to glutathione, and chloramphenicol were used in an attempt to enhance the amination of pyruvate. Chloramphenicol was the more effective agent, and, in further experiments in the presence of this compound, the reductive amination of pyruvate was strongly dependent on DPNH and ATP. A similar diphosphopyridine (DPN) and DPNH dependency has been demonstrated by Wiame and Pierard (1955) for a reversible and specific alanine dehydrogenase system in *Bacillus subtilis*. In another investigation on the amination of pyruvate in *Aerobacter aerogenes*, Fowler and Workman (1955) stipulated the formation of a phosphorylated intermediate.

In the presence of chloramphenicol the optimal pH is 7.2 (Fig. 2) compared with the two maxima shown in Figure 1 (6.9 and 8.2) in the absence of chloramphenicol. These results support the concept that a competitive reaction, retarded by the presence of chloramphenicol, causes the depression in the pH curve.

The reductive amination of pyruvate not only provides the ovary tissue with alanine but, since an active non-specific alanine- α -keto acid transaminase is present

in this tissue (Pollak, unpublished data), a whole array of amino acids may be produced. Furthermore not only is α -amino nitrogen produced, but DPNH is re-oxidized in the process and DPN becomes available for other biological oxidations. As *A. lumbricoides*, along with most parasitic nematodes, is a facultative anaerobe and as there is an excess of pyruvate production over pyruvate oxidation, this substrate may take the part which α -ketoglutaric acid usually plays in aerobic organisms.

The comparison of the effect of lactic acid and ascorbic acid on the reductive amination of pyruvate (Table 3) proved to be very instructive. The results indicate that large amounts of lactate had the same enhancing effect on pyruvate reduction to alanine as had the addition of ascorbic acid. This is in agreement with the evidence of Groth and LePage (1954) who found that ascorbic acid inhibited the reduction of pyruvate to lactate.

This reduction of pyruvate to alanine, dependent on DPNH and ATP, seems to compete with other systems for substrate molecules, and chloramphenicol, ascorbic acid, and excess of lactate all enhance the formation of alanine. The removal of the newly formed alanine, and thus the maintenance of the reaction, seems to be assured by the presence of the non-specific alanine- α -keto acid transaminase as well as by the specific and active alanine-glutamic acid transaminase. Such activity is demonstrated by the small increases of the amino acid spots on the chromatograms. Alanine-glutamic acid transaminase favours glutamic acid formation 2 : 1 over alanine formation from glutamic acid under conditions very similar to those of the present experiments (Pollak and Fairbairn 1955*b*). This equilibrium towards glutamic acid formation, as well as the dialysis procedure which was routinely carried out, exclude the possible formation of alanine from a coupling of the glutamic acid dehydrogenase and alanine-glutamic acid transaminase systems.

The recent work of Wiame and Pierard (1955) showed that in *B. subtilis* oxidative deamination of aspartate, glutamate, and glycine proceeds via a transamination reaction to pyruvate, followed by the deamination of the newly formed alanine. Since the reaction is reversible in this organism there also is a pathway for direct alanine synthesis. Evidence pointing to direct alanine formation has also been obtained by Fairhurst, King, and Sewell (1956) who used 16 different bacterial strains and species.

In the ovary tissue of *A. lumbricoides* the high rate of glycolysis (Rathbone and Rees 1954), the high ammonia content (Pollak and Fairbairn 1955*a*), and the demonstration that ammonia, far from being an excretory product, is utilized *in vivo* for α -amino nitrogen formation (Pollak 1957) as well as the results obtained in the present investigation on the amination of pyruvate, all indicate that pyruvate may well hold a key position for the formation of amino acids in this organism.

V. ACKNOWLEDGMENT

The work reported in this paper was supported by a University of Sydney Research Grant.

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STUDIES IN ANAPHYLAXIS

II. DOSE RESPONSES TO BOVINE PLASMA ALBUMIN IN MICE

By. W. R. SOBEY* and K. M. ADAMS*

[*Manuscript received May 13, 1957*]

Summary

The factors controlling anaphylaxis are discussed.

The optimal shocking dose for bovine plasma albumin was found to be 0.05 mg for both passive and active anaphylaxis.

The relation of optimal shocking dose to the amount of available antibody is discussed.

I. INTRODUCTION

McMaster and Kruse (1949) and Solotorovsky and Winsten (1954) were the first workers to demonstrate that small shocking doses of antigen are just as effective, and often more so, as large doses in producing severe anaphylactic shock in mice. Investigations of the optimal shocking and sensitizing doses of different antigens by Solotorovsky and Winsten (1954) and Cameron (1956) were restricted by the use of an all-or-none score. Since Claringbold and Sobey (1957) developed a quantitative score for different degrees of anaphylaxis it was decided to examine further the relationships of shocking and sensitizing doses to severity of anaphylaxis.

Several factors might influence the severity of an anaphylactic shock. The three factors we have taken into consideration in the experiments described below are as follows:

- (i) The level of antibody made available. In passive anaphylaxis this will depend on the amount of antibody injected; in active anaphylaxis it will depend on the nature of the sensitizing dose and the response of the animal to this dose.
- (ii) The size and route of the shocking dose.
- (iii) The innate susceptibility of the animal to the whole anaphylactic process.

The final outcome, which is an anaphylactic shock of greater or less severity, can be scored. This score is referred to as the *anaphylactic score*. In so far as the score is dependent upon the state of the animal prior to the shocking dose, it will depend upon the extent to which the animal has been primed (by the injection of antibody, or the sensitizing dose of antigen) and its innate susceptibility. This state will be referred to as the animal's *anaphylactic potential*. Different animals having the same level of available antibody may have different anaphylactic potentials because of their individual innate susceptibility to the whole anaphylactic process. This innate susceptibility will be referred to as the animal's *anaphylactic sensitivity*.

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Thus an animal's anaphylactic potential may depend upon the amount of antibody which the experimenter has chosen to inject, or it may depend upon the amount of antibody which the animal has produced in response to a priming dose of antigen, as well as its own anaphylactic sensitivity. An animal's anaphylactic sensitivity depends solely on its innate nature. The anaphylactic score recorded for an animal would depend on the animal's anaphylactic potential at the time a shocking dose was given and the size of the shocking dose.

TABLE 1
DESIGN AND RESULTS OF EXPERIMENT 1
Each treatment combination is the sum of four observations

Shocking Dose (mg)	Sensitizing Dose (mg)				Total
	100	50	25	12.5	
50	152	143	67	38	400
5	143	114	132	96	485
0.5	227	200	221	143	791
0.05	261	215	208	159	843
Total	783	672	628	436	2519

Analysis of Variance

Source of Variation	D.F.	Mean Square	F
Shocking dose	(3)		
Linear	1	8530	41***
Remainder	2	368	2
Sensitizing dose	(3)		
Linear	1	3669	18***
Remainder	2	122	<1
Interactions	9	130	<1
Error	48	209	

*** $P < 0.001$.

In factorially designed experiments, such as those to be described, where animals are randomized to treatment groups, variation between animals will be distributed at random. Thus in the experiments concerned with active anaphylaxis, differences between animals in their ability to produce antibodies and their anaphylactic sensitivity will only contribute to the error term. The variables under study, shocking and sensitizing dose, may be interpreted in terms of anaphylactic score without further regard to differences in anaphylactic potential. In the experiment concerned with passive anaphylaxis, anaphylactic sensitivity is distributed at random but the level of available antibody is one of the variables under study. Therefore,

in terms of anaphylactic score, it is possible to observe changes in anaphylactic potential with different levels of available antibody, and their relation to the other variables studied.

In order to compare different individuals with respect to anaphylactic sensitivity it would be necessary to make available the same amount of antibody to each individual and use a uniform shocking dose.

TABLE 2
DESIGN AND RESULTS OF EXPERIMENT 2
Each treatment combination is the sum of four observations

Shocking Dose (mg)	Sensitizing Dose (mg)				Total
	200	100	50	25	
0.05	194	189	218	130	731
0.005	59	147	118	97	421
0.0005	58	0	58	0	118
Saline	0	29	0	0	29
Total	311	365	394	227	1297

Analysis of Variance			
Source of Variation	D.F.	Mean Square	F
Shocking dose	(3)		
Linear	1	18165.4	39.8***
Remainder	2	459.3	1
Sensitizing dose	3	336.6	<1
Interactions	9	229.3	<1
Error	48	455.5	

*** $P < 0.001$.

II. MATERIAL AND METHODS

(a) Mice

Adult female albino mice, weighing about 20 g, obtained from a randomly bred strain were used in this work. The mice were given standard mouse cubes and water *ad lib*.

(b) Antigen

Bovine plasma albumin (B.P.A.) (Armour fraction V) was used throughout. In all experiments requiring active sensitization, half the total dose was given in each of two intraperitoneal injections which were spaced by a week. The shocking injections were administered intravenously, and, except where otherwise stated, were given 1 week after the last sensitizing injection. All injections were of 0.5 ml.

(c) Preparation of Antiserum

High-titre antiserum was prepared from selected rabbits which received a total of 16 bi-weekly intravenous injections of B.P.A. This antiserum gave an optimal proportions reading of 9 on the scale given by Sang and Sobey (1954), i.e. the first tube to flocculate contained equal volumes of antiserum and B.P.A., the final concentration of the latter being 0.1 per cent.

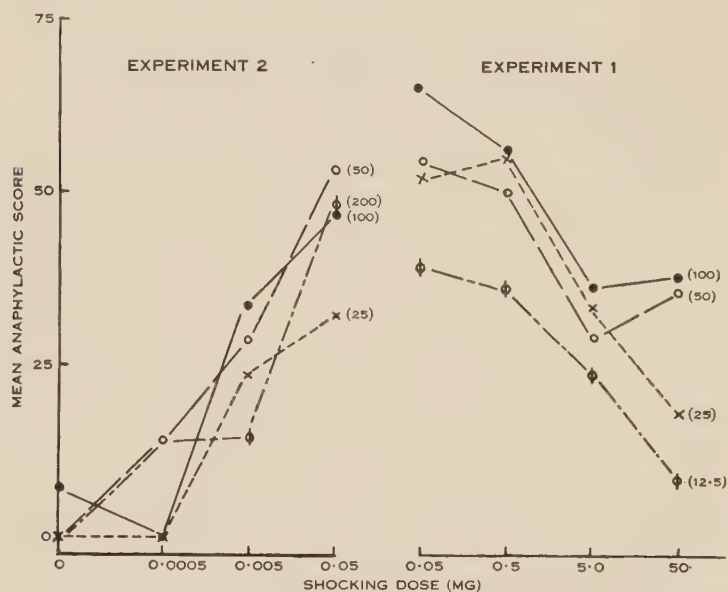


Fig. 1.—Mean anaphylactic score is plotted against shocking dose for experiments 1 and 2. Sensitizing dose (in mg) is shown in parenthesis for each response line.

(d) Quantitative Score

The score as described by Claringbold and Sobey (1957) was used throughout, namely:

Score	Description of Symptoms
0	No effect.
29	Animal exhibits vigorous scratching of ears and face, and nibbling of the anal region.
38	Animals displays absence of any desire to move, huddles in a corner, with laboured breathing and infrequent convulsive movements. The animal will move when stimulated.
59	Paralysis, partial or complete. The animal does not move when stimulated or moves slowly, dragging its hindquarters.
65	Death.

The value given to each symptom was arrived at by discriminant analysis.

Two observers scored each experiment independently without pre-knowledge of the treatments accorded the mice under observation. The repeatability of scoring was found to be high ($r = 0.76$, $z = 0.9 \pm 0.09$) and a mean of the two scores was used.

TABLE 3
DESIGN AND RESULTS OF EXPERIMENT 3
Each treatment combination is the sum of five observations

Shocking Dose (mg)	Sensitizing Dose (mg)					Total
	200	100	50	25	12.5	
50	87	116	58	58	29	348
5	134	227	199	153	146	859
0.5	277	226	256	241	195	1195
0.05	199	292	286	313	271	1361
0.005	244	292	184	229	325	1274
Total	941	1153	983	994	966	5037

Analysis of Variance

Source of Variation	D.F.	Mean Square	<i>F</i>
Shocking dose	(4)		
Linear	1	22165.2	72.7***
Quadratic	1	5331.3	17.4***
Cubic	1	24.3	<1
Quartic	1	4.4	<1
Sensitizing dose	4	280.9	<1
Interactions	16	356.8	1.2
Error	100	304.8	

*** $P < 0.001$.

(e) Experimental Design

All the experiments described are of factorial design (Cochran and Cox 1950) and since the treatment variables, sensitizing and shocking dose, are not constant over all experiments they are detailed for each experiment in the text.

III. RESULTS

(a) Active Anaphylaxis

(i) *Experiment 1*.—This was a 4^2 factorial experiment with four mice in each treatment group. The two variables studied, sensitizing and shocking dose, were each given at four different levels. The experimental design and the results obtained are given in Table 1 together with the analysis of variance of these results. Anaphylactic score increased log-linearly with increased sensitizing dose, suggesting a

dependence on the quantity of antibody produced. Anaphylactic score decreased log-linearly with increased shocking dose. Neither response line showed an inflexion point and a further experiment was undertaken to extend the range of both variables.

(ii) *Experiment 2*.—Apart from the size of the shocking and sensitizing doses this experiment was identical with the previous one. The design and results and their analysis of variance are given in Table 2. The size of the sensitizing dose had no significant effect on anaphylactic score, suggesting that doses of 25 mg and above do not significantly increase the antibody response. Anaphylactic score increased log-linearly with increased shocking dose. In both this and the previous experiment the optimal shocking dose was 0.05 mg. One animal out of 16 injected with saline

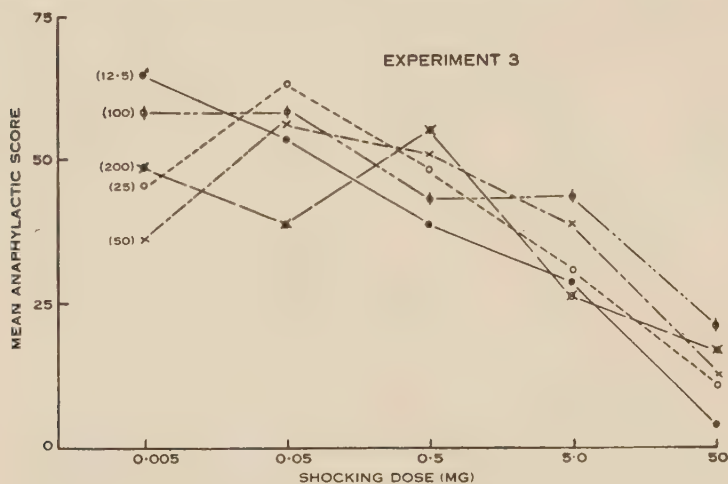


Fig. 2.—Mean anaphylactic score is plotted against shocking dose for experiment 3. Sensitizing dose (in mg) is shown in parenthesis for each response line.

was scored 29. This frequency of mis-scoring of this symptom would have little effect on the results. The two experiments are plotted together in Figure 1 from which it appears that increasing sensitizing dose at all levels of shocking dose has a relatively slight effect beyond 25 mg.

(iii) *Experiment 3*.—To confirm the previous findings a larger experiment was undertaken. This was a 5^2 factorial experiment with five mice in each treatment group, i.e. a total of 125 mice. The design and results are given in Table 3 together with their analysis of variance. Anaphylactic score increased log-linearly with decreased shocking dose to 0.05 mg after which it decreased confirming 0.05 mg as the optimal shocking dose. The size of the sensitizing dose had no apparent effect on anaphylactic score. The scores reached (Fig. 2) are similar, though not identical with experiments 1 and 2, and, although in each the optimum shocking dose is at 0.05 mg, there is some indication that it might be less where sensitizing doses are less.

(b) *Passive Anaphylaxis*

(i) *Experiment 4*.—This was a $3^2 \times 2$ factorial experiment with four mice in each treatment group, i.e. 72 mice in all. The sensitizing dose was an 0.5 ml

intraperitoneal injection of rabbit anti-B.P.A. diluted in normal saline and was given at three levels. The shocking dose was given at three levels, and the shocking was carried out at two different times after sensitizing.

Olitsky and Lee (1955) used up to 2 ml of neat rabbit serum in intraperitoneal injections to sensitize mice passively. We found our rabbit serum to be toxic to

TABLE 4
DESIGN AND RESULTS OF EXPERIMENT 4
Each treatment combination is the sum of four observations

Shocking Dose (mg)	Levels of Sensitizing Dose								Total
	24 Hr before Shocking				48 Hr before Shocking				
	1/5	1/25	1/125	Total	1/5	1/25	1/125	Total	
0.5	176	117	58	351	134	87	87	308	659
0.05	242	125	87	454	242	125	87	454	908
0.005	116	116	116	348	87	87	125	299	647
Total	534	358	261	1153	463	299	299	1061	2214

Analysis of Variance

Source of Variation	D.F.	Mean Square	F
Shocking dose (A)	(2)		
Linear	1	3.0	1
Quadratic	1	1806.0	12.1***
Sensitizing dose (B)	(2)		
Linear	1	3978.5	28.3***
Quadratic	1	410.1	2.1
Times of shocking (C)	1	117.6	<1
First-order interactions			
A × B	4	1001.4	7.1***
A × C	2	29.5	<1
B × C	2	144.4	1.1
Second-order interactions			
A × B × C	4	45.9	<1
Error	54	140.4	

*** $P < 0.001$.

about 15 per cent. of our randomly bred mice if given neat in doses of 0.5–1 ml intraperitoneally, but non-toxic when diluted 1/5 in normal saline. The serum had a Forssman titre of 1/64 indicating the reason for the deaths. Absorption of the neat serum by sheep red cells reduced the number of deaths; when 0.5 ml of absorbed serum was given intraperitoneally no deaths out of 24 mice occurred whereas three deaths out of 24 occurred without absorption. Given intravenously, one death out

of 10 occurred when absorbed serum was injected, and three deaths out of 10 without absorption. While these data suggest that absorption may reduce the number of deaths they are not statistically significant. To avoid losses, the highest concentration used in the experiment was a 1/5 dilution in saline.

The design and results of the experiments and the analysis of variance are given in Table 4. Anaphylactic score increases log-linearly with increased antibody concentration. The response line to shocking dose is quadratic with the inflexion point at a dose of 0.05 mg, the same optimal as found in the previous experiment. The significant interaction between shocking dose and sensitizing dose appears to be due to markedly quadratic response to the sensitizing dose of 1/5 and the more linear responses to 1/25 and 1/125 (Fig. 3). This indicates a shift of the optimal shocking dose with the lowering of sensitizing dose, a trend of which there was some suggestion in the experiments on active anaphylaxis (Figs. 1 and 2).

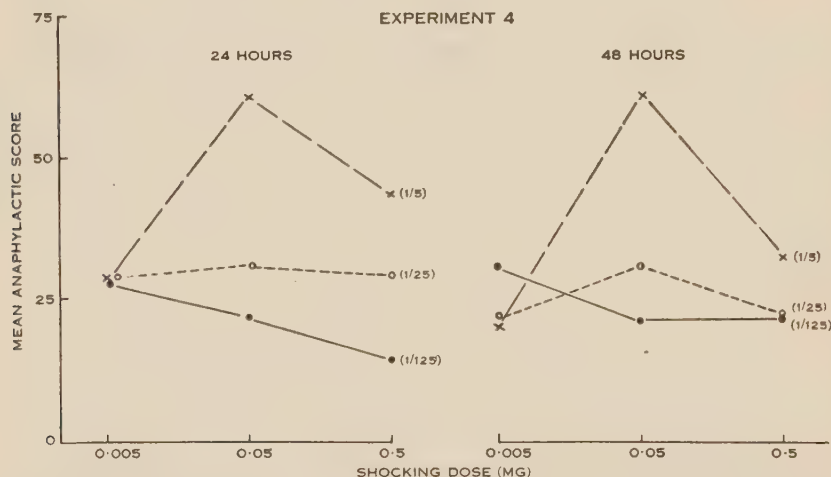


Fig. 3.—Mean anaphylactic score is plotted against shocking dose for experiment 4. Level of sensitizing dose is shown in parenthesis for each response line for both 24 and 48 hr after sensitizing.

IV. DISCUSSION

It is seen from Table 4 that anaphylactic score increases log-linearly with the amount of antibody made available, demonstrating the dependence of anaphylactic potential upon the level of available antibody. Pittman and Germuth (1954), using two rabbit anti-B.P.A. sera containing 0.816 and 2.52 mg antibody nitrogen respectively, found that deaths due to anaphylaxis were consistently higher where the serum containing the most antibody nitrogen was used, although they were unable to demonstrate a statistically significant difference.

The first three experiments, involving active anaphylaxis, all indicate the optimal shocking dose to be in the region of 0.05 mg. This same dose was found to be optimal for shocking in passive anaphylaxis. While there is some suggestion that the optimal shocking dose may change with changing anaphylactic potential this is not marked, and, on the average, 0.05 mg is superior to any other shocking dose

over a very wide range of treatments. The absence of an optimal antigen-antibody ratio, in respect of shocking dose to available antibody, is at present inexplicable.

V. ACKNOWLEDGMENTS

We wish to thank Dr. J. M. Rendel for his helpful criticism of the manuscript and Miss Pam Back for her technical assistance.

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SIMULATION OF GENETIC SYSTEMS BY AUTOMATIC DIGITAL COMPUTERS

I. INTRODUCTION

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[Manuscript received June 26, 1957]

Summary

Methods of setting automatic digital computers to simulate the algebraic aspects of reproduction, segregation, and selection are discussed. The application of these methods to the problem of the importance of linkage in multifactorial inheritance is illustrated by results from the SILLIAC.

I. INTRODUCTION

In recent years a field of mathematics has become prominent which is based on the simulation of stochastic processes. This field has been termed the Monte Carlo method and its prominence can be directly attributed to the introduction of automatic electronic digital computers. The Monte Carlo method involves in most of its applications several hundreds of thousands of arithmetic steps and therefore would be impractical without the speed of automatic computers.

The majority of genetic problems depend for their resolution on the algebra of repetitive sequences, and it is relatively easy to apply the Monte Carlo method to these sequences. The general problem of applying the Monte Carlo method to genetic problems using an automatic digital computer (the SILLIAC) is discussed in this paper.

II. BINARY REPRESENTATION OF GENETIC FORMULA

If we consider two alleles, $+^a$ and a , at a locus, the genotypes are represented as $+^a +^a$, $+^a a$, and aa respectively, and the gametes as $+^a$ and a . In this system $+$ and *not-plus* specify the two alleles, a specifies the locus. The symbols 1 and 0 can

TABLE 1
NORMAL AND BINARY REPRESENTATION OF TWO HAPLOID GENOTYPES

Normal	$+^a$	b	c	d	$+^e$	f	g	$+^h$	$+^i$	$+^j$	$+^k$	l	m
Binary	1	0	0	0	1	0	0	1	1	1	1	0	0
Normal	$+^a$	$+^b$	$+^c$	$+^d$	$+^e$	f	g	h	i	j	k	$+^l$	$+^m$
Binary	1	1	1	1	1	0	0	0	0	0	0	1	1

be substituted for $+$ and *not-plus*, and the position of the symbols in a register can specify the locus. This is illustrated in Table 1 for two haploid genotypes.

Since it is possible to manipulate the individual digits of a register, this binary arithmetical representation of genetic formulae is the first step in simulating genetic systems.

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III. "LOGICAL" ALGEBRA

A special aspect of the circuits of the ILLIAC family of computers is the ability to perform the operations of "logical" algebra. These are illustrated below:

$$\begin{array}{ll} 0\ 1\ 1 \ \& \ 0\ 0\ 1 = 0\ 0\ 1 & \text{Logical product} \\ 0\ 1\ 1 \ \equiv \ 0\ 0\ 1 = 0\ 1\ 0 & \text{Logical equivalent} \\ 0\ 1\ 1 \ \wedge \ 0\ 0\ 1 = 1\ 0\ 0 & \text{Logical not-sum} \end{array}$$

These operations can be used to allow identification of the genetic nature of an individual at each locus, i.e. whether the individual is $+^i+$, $+^ii$, or ii at a locus i . Given two haploid genotypes A (paternal) and B (maternal), then

$A \ \& \ B$ identifies the $++$ loci,

$A \ \equiv \ B$ identifies the $+^ii$ loci,

$A \ \wedge \ B$ identifies the ii loci.

This is illustrated for a diploid genotype of 10 loci:

$$\begin{array}{rcl} A \text{ (paternal genotype)} & 1\ 0\ 1\ 1\ 0\ 0\ 0\ 1\ 1\ 0 & \\ \hline B \text{ (maternal genotype)} & 1\ 1\ 0\ 1\ 1\ 0\ 0\ 1\ 0\ 1 & \\ A \ \& \ B & = 1\ 0\ 0\ 1\ 0\ 0\ 0\ 1\ 0\ 0 \\ A \ \equiv \ B & = 0\ 1\ 1\ 0\ 1\ 0\ 0\ 0\ 1\ 1 \\ A \ \wedge \ B & = 0\ 0\ 0\ 0\ 0\ 1\ 1\ 0\ 0\ 0 \end{array}$$

The importance of these transformations is most evident in the determination of the phenotypic value corresponding to a specific genotype.

IV. DETERMINATION OF PHENOTYPIC VALUE

If the contribution of the homozygous recessive loci to the phenotype is taken as zero it is only necessary to specify the phenotypic component of the homozygous dominant loci and the dominance term. If a is the phenotypic component and h is the dominance term, then the phenotypic contribution of a locus is 0, ah , or a , depending on whether it is 0/0, 0/1, or 1/1.

In the general case each locus may have a different phenotypic component and dominance term, and the vectors $\{a_i\}$ and $\{h_i\}$ need to be specified. If these be considered as diagonal matrices, and the logical product and logical sum of A and B be also considered as diagonal matrices (where A is the maternal and B the paternal genotype), then the phenotype of the individual is given by

$$\text{diag } A \ \& \ B \cdot \text{diag } \{a_i\} + \text{diag } A \ \equiv \ B \cdot \text{diag } \{a_i\} \cdot \text{diag } \{h_i\} = \text{diag } [P_i],$$

and

$$P_{AB} = \Sigma p.$$

This is illustrated for a genotype of four loci, in which $\{a_i\}$ is $\{1, 2, 3, 4\}$ and $\{h_i\}$ is $\{1.0, 0.5, 0.25, 0.0\}$. Using binary notation, $A = 1\ 0\ 1\ 0$, and $B = 1\ 1\ 0\ 0$. Then $\text{diag } A \ \& \ B \cdot \text{diag } \{a_i\} =$

$$\begin{bmatrix} 1 & . & . & . \\ . & 0 & . & . \\ . & . & 0 & . \\ . & . & . & 0 \end{bmatrix} \begin{bmatrix} 1 & . & . & . \\ . & 2 & . & . \\ . & . & 3 & . \\ . & . & . & 4 \end{bmatrix} = \begin{bmatrix} 1 & . & . & . \\ . & 0 & . & . \\ . & . & 0 & . \\ . & . & . & 0 \end{bmatrix} = C,$$

and $\text{diag } A \equiv B \cdot \text{diag } \{a_i\} \cdot \text{diag } \{h_i\} =$

$$\begin{bmatrix} 0 & . & . & . \\ . & 1 & . & . \\ . & . & 1 & . \\ . & . & . & 0 \end{bmatrix} \begin{bmatrix} 1 & . & . & . \\ . & 2 & . & . \\ . & . & 3 & . \\ . & . & . & 4 \end{bmatrix} \begin{bmatrix} 1.0 & . & . & . \\ . & 0.5 & . & . \\ . & . & 0.25 & . \\ . & . & . & 0.0 \end{bmatrix} = \begin{bmatrix} 0 & . & . & . \\ . & 1 & . & . \\ . & . & 0.75 & . \\ . & . & . & 0 \end{bmatrix} = D,$$

and

$$C + D = \begin{bmatrix} 1 & . & . & . \\ . & 1 & . & . \\ . & . & 0.75 & . \\ . & . & . & 0 \end{bmatrix} = [P_i],$$

from which

$$P_{AB} = \Sigma p = 2.75.$$

The steps of this sequence can easily be programmed and the required space in the memory is small: two sets of n registers where n is the number of loci.

V. INTER-LOCUS INTERACTIONS

It is necessary for the completely general case to include inter-locus interactions. This can be done by specifying three matrices of order n , each matrix specifying the occurrence and order of interactions for each genetic state, i.e. separate matrices are required for the 0/0, 0/1, and 1/1 states. The space required to store these matrices would severely restrict the usefulness of a programme except for very few loci. This restriction can be avoided by restricting the number of degrees of interaction to a reasonable number, say 3. Here the required storage space would be 27 registers for the three matrices, and $2n^2$ digits for storage of the matrix specifying whether interaction occurs, and if so, of what type.

In the following matrix the rows specify the locus whose phenotype is modified by interaction, and the columns specify the locus performing the interaction. The elements of the matrix are modulo₄, i.e. 0, 1, 2, or 3, and specify, if 0, that no interaction occurs, if 1, 2, 3, that interaction does occur and is of type 1, 2, or 3. If n is 20, then only 20 registers are required to store this matrix which is a minor demand on the memory space:

$$\begin{bmatrix} b_{00} & . & . & . & b_{0n} \\ . & . & . & . & . \\ . & . & . & . & . \\ . & . & . & . & . \\ . & . & . & . & . \\ b_{n0} & . & . & . & b_{nn} \end{bmatrix} \text{ modulo}_4$$

These sequences produce a single-valued phenotype. It is possible, by paralleling to produce a multi-valued phenotype, thus allowing simulation of genetic systems involving several characters.

VI. ENVIRONMENTAL EFFECTS

The above method of determining the phenotype corresponding to a specific genotype does not include any effect of non-genetic factors. Since the majority of the unsolved problems of mathematical genetics occur in systems with environmental modification of the phenotype it is necessary to simulate the occurrence of an environmental component of the phenotype which is independent of the genotype.

This can be accomplished by specifying a function $r = f(x)$ such that if r is a random number in the range 0 to 1, then x is a random normal deviate also in the range -1 to $+1$. Hastings (1955) has devised several functions which, using linear combinations of r , produce values corresponding closely to random normal deviates.

Given that P_i is the potential phenotype of the i th genotype, then by generating a random number, r_i , and finding x_i , the transformation of the potential phenotype into the actual phenotype is given by

$$[P_i]_{\text{actual}} = P_i \pm x_i \cdot P_i.$$

It is clearly possible, by specifying different forms of $r = f(x)$, to simulate any degree of environmental modification of the potential phenotype. It is also possible to specify relations between the genotype and $r = f(x)$, i.e. to simulate genetic control of environmental stability. This is likely to be an important feature of programmes designed to examine systems showing "homeostasis" (Lerner 1954).

VII. SEGREGATION

Gametes produced by an individual heterozygous for a single locus are of two types, both occurring with equal probability: e.g. the gametes produced by the heterozygote 0/1 are of the types 0 and 1 with equal probability. This process can be simulated by generating a random number, r_i , which lies in the range

$$0 \leq r_i \leq 1.$$

If this number is tested against 0.5, then, in a set of q random numbers, the occurrence of tests which exceed 0.5 will have the same probability as those which are less than 0.5, allowing for slight bias introduced by the accuracy of the random number. If the random numbers have an accuracy of 1×2^{-38} , as is usual in the SILLIAC, then any bias of this system is negligible.

This method can be used to simulate a system of n genetically independent loci. Given that A and B represent the paternal and maternal genotypes of an individual as before, then the following sequence will simulate the production of a gamete by such an individual.

Form $A \& B$ and $A \equiv B$. Operate on $A \equiv B$ in digital sequence, generating a random number, and testing it against 0.5 (as above) for each digital position which contains 1. The result is that $A \equiv B$ will be transformed into a term

$\langle A \equiv B \rangle$ in which the digital positions containing 0 are unaffected, whereas those containing 1 are left alone or changed to 0 with equal probabilities which are independent between positions. If we represent the operation of the random transform by $\bar{R}$, then this is expressed by

$$\bar{R} : A \equiv B \rightarrow \langle A \equiv B \rangle.$$

Then $A \& B + \langle A \equiv B \rangle$ gives a number whose digital conformation is such that (i) it has 0 wherever the AB configuration was 0/0, (ii) it has 1 wherever the AB configuration was 1/1, and (iii) it has 1 or 0 at equal probability wherever the AB configuration was 0/1 or 1/0.

VIII. RECOMBINATION

The simulation of recombination can be accomplished given the vector of frequencies of recombinant and non-recombinant classes. The vector is illustrated below for the gametes produced by an individual heterozygous at three loci, in coupling, where r_1 is the recombination between the first and second loci, and r_2 is the recombination between the second and third loci.

Vector of Types of Gametes		Frequencies	
$\frac{1\ 1\ 1}{0\ 0\ 0}$ produces (individual's genotype)	0 0 0	$\frac{1}{2} (1-r_1) (1-r_2)$	$= f_{000}$
	0 0 1	$\frac{1}{2} (1-r_1) r_2$	$= f_{001}$
	0 1 0	$\frac{1}{2} r_1 r_2$	$= f_{010}$
	0 1 1	$\frac{1}{2} r_1 (1-r_2)$	$= f_{011}$
	1 0 0	$\frac{1}{2} r_1 (1-r_2)$	$= f_{100}$
	1 0 1	$\frac{1}{2} r_1 r_2$	$= f_{101}$
	1 1 0	$\frac{1}{2} (1-r_1) r_2$	$= f_{110}$
	1 1 1	$\frac{1}{2} (1-r_1) (1-r_2)$	$= f_{111}$

This illustration has been given for a triple heterozygote, but $\{f_i\}$, the vector of frequencies of recombinants and non-recombinants, is not restricted to a particular genotype. The same distribution of frequencies of recombinants and non-recombinants occurs for all genotypes. The effect of considering a different genotype is to change the vector of types of gametes. This is illustrated for three genotypes:

Vector of Types of Gametes		
$\frac{1\ 0\ 1}{0\ 1\ 0}$ produces	1 0 1	1 1 0
	1 0 0	1 1 1
	1 1 1	1 0 0
	1 1 0	0 0 0
	0 0 1	1 1 0
	0 0 0	1 1 1
	0 1 1	1 0 0
	0 1 0	1 0 1
$\frac{1\ 1\ 0}{1\ 0\ 0}$ produces		0 0 0
$\frac{0\ 0\ 0}{0\ 0\ 0}$ produces		0 0 0

The sequence of operations necessary to simulate the production of gametes by an individual of any genotype is shown below, given that the genotype of the individual is abc/def and that $\{f_i\}$ is the vector of frequencies of recombinants and non-recombinants. The first step is to form the vector of types of gametes as shown:

Vector of Types of Gametes	
	$a\ b\ c$
	$a\ b\ f$
	$a\ e\ c$
	$a\ e\ f$
abc	produces
$\overline{def}$	
	$d\ b\ c$
	$d\ b\ f$
	$d\ e\ c$
	$d\ e\ f$

The second step is to transform $\{f_i\}$ by sequential summation to give

$$\{f_{000}; f_{000}+f_{001}; f_{000}+f_{001}+f_{010}; \dots \Sigma f\}.$$

It is convenient to set $\{f_i\}$ such that $\Sigma f = 1$. The vector produced by sequential summation will be termed the $\{F_i\}$ vector.

A random number, r_i , is then generated such that

$$0 \leq r_i \leq 1.$$

This number is then tested across $\{F_i\}$ until

$$F_i < r_i < F_{i+1}.$$

Then the i th term in the vector of types of gametes is taken as the gamete produced. Repetition of this sequence will produce a number of gametes in which the various types occur with probabilities corresponding to the frequencies of recombinants and non-recombinants.

The simulation of the formation of a gamete, g , by an individual of genetic constitution A/B is represented as

$$T : A/B \rightarrow g,$$

where T represents the operation of forming the vector of types of gametes, testing a random number against $\{F_i\}$, and then selecting the i th type of gamete from the vector of types of gametes.

This method of simulating recombination can be set to simulate the independent assortment of linkage groups by specifying one or more of the values of r to be 0.5. If the genetic system to be simulated is of six loci then the following values of r will simulate two linkage groups:

Location	0	1	2	3	4	5
r	0.1	0.1	0.5	0.1	0.1	

A major limitation of this method is that 2^n registers, where n is the number of loci, are required to store $\{F_i\}$. This limitation can be reduced by simulating the formation of each linkage group separately. If linkage groups are numbered from 0 to N , then the operation can be represented as

$$T : A_i/B_i \rightarrow g_i,$$

where $i = 0$ to N . Then, since each of the transforms of this transformation are independent, the vector of "chromosomes", i.e. $\{g_i\}$, simulates the independent assortment of linkage groups and the occurrence of linkage within each linkage group. A major advantage of this system is that the number of loci is Nn' , where n' is the number of loci per linkage group. The required space in the memory is then $N2^{n'}$, which is considerably less than $2^{Nn'}$; further, if the restriction that the linkage relations are the same for each linkage group be accepted, then the required storage is $2^{n'}$, and any restrictions of number of loci are imposed by the time necessary for calculation rather than by the size of the memory.

The sequences discussed above allow the simulation of (i) the formation of a set of genotypes, $\{A/B\}_{\text{progeny}}$, from a set of parental genotypes, $\{A/B\}_{\text{parents}}$, and (ii) the formation of a set of phenotypes, $\{p\}_{\text{progeny}}$, from the genotypes of the progeny, $\{A/B\}_{\text{progeny}}$.

IX. SELECTION

There are several methods of simulating selection, the majority being variations of the following sequence. The first step is to re-order the phenotypes of the progeny, i.e. $\{p\}_{\text{progeny}}$, in ascending or descending sequence, re-ordering the genotypes of the progeny correspondingly. Then, if there are N' progeny and the programme specifies that N'' of these be retained as parents, it is possible to take (i) the N'' genotypes of $\{A/B\}_{\text{progeny}}$ with the greatest phenotypes, (ii) the N'' genotypes of $\{A/B\}_{\text{progeny}}$ with the least phenotypes, or (iii) the N'' genotypes of $\{A/B\}_{\text{progeny}}$ with phenotypes closest to the mean. These three alternatives simulate selection for one or other extreme or against extremes.

An important aspect of re-ordering the set of genotypes of progeny is that, where phenotypes are identical, position in that section of the store be kept at random, since, where the programme specifies the formation of many progeny per mating, it is possible, without such a precaution, for selection to favour a specific mating.

A deficiency of this method of simulating the operation of selection is that no account is taken of the variation of the number of progeny produced per individual. This can be included by a variation of the method of random normal deviates discussed above. If r_i is a random number and $r_i = (x)$, where x is a random normal deviate in the range 0 to 1, then, given q , the potential number of progeny, it is possible to modify this by

$$q \cdot x_i = Q_i,$$

where Q_i is the actual number of progeny for the i th individual.

Clearly many variations are possible. An example is to relate q to the potential or actual phenotype. This is accomplished by setting $q = f(P_i)_{\text{potential}}$, or $q = f(P_i)_{\text{actual}}$. A limitation of all these more sophisticated models is that the time necessary for calculation is increased, sometimes markedly, over the simpler models.

X. CONCLUSIONS

The sequences discussed above, when formulated in a programme suitable for an automatic computer, allow simulation of genetic systems. There are various problems in which this approach may be useful. These are (i) the effects of linkage on the efficiency of selection, (ii) the construction of tables relating the competitive efficiencies of alleles to the parameters of population size, intensity of selection, etc., and (iii) the comparison of the efficiencies of different breeding plans for varying degrees of inter-locus interactions. In subsequent papers of this series results gained from running various programmes covering these problems will be discussed.

XI. ACKNOWLEDGMENTS

I am deeply indebted to Dr. P. J. Claringbold for introducing me to the Monte Carlo method, and for his patient tuition in various aspects of programming. The staff of the Adolph Basser Computing Laboratory were extremely helpful, particularly Dr. J. C. Bennett, Dr. B. Chartres, and Mr. J. Butcher. I am grateful to Miss J. Ogilvie for her preparation and meticulous checking of the various programmes.

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SIMULATION OF GENETIC SYSTEMS BY AUTOMATIC DIGITAL COMPUTERS

II. EFFECTS OF LINKAGE ON RATES OF ADVANCE UNDER SELECTION

By A. S. FRASER*

[Manuscript received July 4, 1957]

Summary

Rates of progress of single populations under selection pressure have been simulated by an automatic electronic computer. Varying intensities of selection and tightness of linkage are compared, showing that linkage produces no qualitative effect on the rates of advance at values greater than 0.005, i.e. 0.5 per cent. recombination.

I. INTRODUCTION

Several of the sequences discussed in the introductory paper of this series (Fraser 1957) have been used as the basis of a programme which simulates the effect of selection on a single population of cross-breeding organisms, in which there is no overlap of generations. The programme is based on a genetic system of six loci, and provision has been made to allow variation of the recombination between loci from $r = 0.5$ to $r = 0$, i.e. from independent assortment to complete linkage. It is therefore possible using this programme to produce results which simulate the rates of advance under selection for different degrees of linkage.

II. STRUCTURE OF THE PROGRAMME

The general sequence of the programme is as follows:

- (i) *Given* the recombination values, form the vector of frequencies of recombinants and non-recombinants; this is termed $\{f_i\}$.
- (ii) *Given* the vector of frequencies of types of haploid genotypes (i.e. types of gametes) which is termed $\{g_i\}$, then this vector is set to sum to unity, i.e.

$$\Sigma g = 1.$$

- (iii) Transform $\{g_i\}$ by sequential summation, i.e. transform $\{g_i\}$ to $\{\hat{g}_i\}$, on the operation

$$\hat{g}_i = \sum_0^i g.$$

- (iv) Generate a random number, S_j , and test this against $\{\hat{g}_i\}$ by counting the number of times that $S_j > \hat{g}_i$ until $\hat{g}_{i+1} \geq S_j > \hat{g}_i$.

Take the binary digital formula of i as the genetic structure of a haploid gamete; term this a_0 . Repeat to produce a second gamete; term this a_1 .

Then $\{a_0, a_1\}$ is the genetic structure of a diploid genotype, i.e. of a single progeny.

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(v) Repeat sequence (iv) N times, producing a set of N diploid genotypes, i.e. produce N progeny. This is termed $[\{a_0 \cdot a_1\}]$.

(vi) Determine the logical sum of a_0 and a_1 . Sum the digits of the logical sum to give the phenotype. This is repeated for the N separate diploid genotypes, giving a set of N phenotypes corresponding to the set of N diploid genotypes; term this $\{P_i\}$.

(vii) Re-order $\{P_i\}$ in terms of descending value, re-sorting $[\{a_0 \cdot a_1\}]$ to correspond.

(viii) Take the first N' values from $[\{a_0 \cdot a_1\}]$ as the parents of the next generation.

(ix) Take $\{a_0 \cdot a_1\}_i$, and form the vector $[a_0/a_1]_{2^n}$ as detailed in the recombination section of the introductory paper to this series (Fraser 1957). This vector is of the types of gametes produced by a_0/a_1 , and the relative frequencies of the types of gametes is given by $\{f_i\}$, i.e. the frequency of the i th term in the vector of types of gametes is f_i .

(x) Each individual is assumed to form the same number of gametes; therefore, if the number of parents is N' and the vector of frequencies of types of gametes, $\{f_i\}$, is divided by N' , and each term is added to $\{g_i\}$, the vector of frequencies of types of gametes produced by N' parents is in the position specified by $[a_0/a_1]$. This is illustrated for a genetic system of two loci:

Given

$$a_0 = 0 \ 1,$$

and

$$a_1 = 1 \ 0,$$

and

$$\{f_i\} = \begin{Bmatrix} 0.3 \\ 0.2 \\ 0.2 \\ 0.3 \end{Bmatrix},$$

i.e. $r = 0.2$, then

$$[a_0/a_1] = \begin{bmatrix} 0 & 1 \\ 0 & 0 \\ 1 & 1 \\ 1 & 0 \end{bmatrix},$$

and if $N' = 10$, then

$$\{g_i\} = \begin{Bmatrix} 0.02 \\ 0.03 \\ 0.03 \\ 0.02 \end{Bmatrix}.$$

(xi) Repeating these sequences gives a new set of values of $\{g_i\}$. If we specify the whole of the above sequence as a transform $\bar{S}$, then

$$\bar{S} : \{g_i\}_j \rightarrow \{g_i\}_{j+1},$$

and the general sequence is

$$\bar{S} : \{g_i\}_0 \rightarrow \{g_i\}_1 \rightarrow \{g_i\}_2 \rightarrow \dots \rightarrow \{g_i\}_j,$$

expressing the continued transformation of the genetic structure of the original population.

III. INFORMATION GENERATED BY THE PROGRAMME

The programme was set to print certain information for each generation, namely $\{g_i\}_j$, $\bar{P}_j$, and σ_p^2 . Each run of the programme gave values simulating the rate of advance under selection over a period of 20–25 generations. Runs were made covering twenty variations of the genetic constants. Two runs were made at each set of values of the genetic constants, starting at different points in the random number sequence, thus giving two replicates.

IV. VALUES OF THE GENETIC CONSTANTS

The genetic structure of the original population, i.e. $\{g_i\}_0$, was set at two values which are termed the P and Q values. They were set as shown below.

(a) P Value of $\{g_i\}_0$

Only those types of gametes listed below were represented in the original population.

Type of Gamete	Frequency
0 1 1 0 1 1	0.1111
0 1 1 1 0 1	0.1111
0 1 1 1 1 0	0.1111
1 0 1 0 1 1	0.1111
1 0 1 1 0 1	0.1111
1 0 1 1 1 0	0.1111
1 1 0 0 1 1	0.1111
1 1 0 1 0 1	0.1111
1 1 0 1 1 0	0.1111

It can be seen that if the six loci are separated into two linkage groups of three loci, then this specification of the genetic structure of the original population is such that two cross-overs must occur before individuals homozygous for the 1 type of allele can occur.

This programme does not allow any variation of the dominance relations, being fixed on complete dominance of the 1 alleles. The P value of $\{g_i\}_0$ therefore specified, if the phenotypic component of the 1 allele at each locus is 1.0, that the mean phenotype of the original population was 5.2. Since the maximum phenotype is 6.0, the runs with the value of $\{g_i\}_0$ are essentially on a quantal basis.

(b) *Q Value of $\{g_i\}_0$*

Only those types of gametes listed below were represented in the original population.

Type of Gamete	Frequency
0 0 1 0 0 1	0.1111
0 0 1 0 1 0	0.1111
0 0 1 1 0 0	0.1111
0 1 0 0 0 1	0.1111
0 1 0 0 1 0	0.1111
0 1 0 1 0 0	0.1111
1 0 0 0 0 1	0.1111
1 0 0 0 1 0	0.1111
1 0 0 1 0 0	0.1111

The second value of $\{g_i\}_0$ is such that four cross-overs must occur before individuals homozygous for the 1 type of allele can occur. The mean of the original phenotype was 3.2.

The 20 runs of the programme, each replicated, are therefore divided into two sets of 10 runs: one with the *P* value of $\{g_i\}_0$, the other with the *Q* value of $\{g_i\}_0$.

The runs were made at two sizes of population. The first value was set to correspond to a medium-sized population with a low intensity of selection (of the order of selection experiments with larger animals). The number of progeny, *N*, was set at 100, the number of parents, *N'*, at 50. The second value was set to correspond to a small population with a high intensity of selection (of the order of selection experiments with *Drosophila*). The number of progeny was set at 40, the number of parents at 4. These two values of the size of population are termed the "large" and "small" values respectively. Runs of the programme were made at five different values of the linkage relations of the six loci. These are listed below:

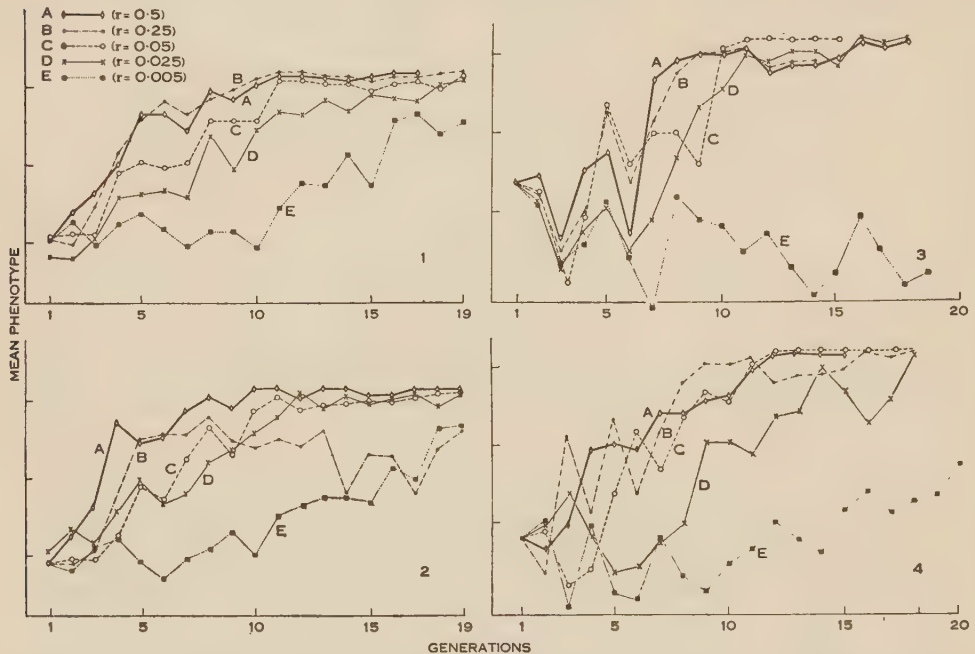
Linkage	Locus					
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>
<i>A</i>	0.5	0.5	0.5	0.5	0.5	
<i>B</i>	0.25	0.25	0.5	0.25	0.25	
<i>C</i>	0.05	0.05	0.5	0.05	0.05	
<i>D</i>	0.025	0.025	0.5	0.025	0.025	
<i>E</i>	0.005	0.005	0.5	0.005	0.005	

The A value of the linkage relations specifies genetic independence of the loci, and the other values specify various degrees of linkage of the loci within two "chromosomes" of three loci, i.e. abc and def .

V. RESULTS

(a) The P Set of Runs

The results of the runs at the P value of $\{g_i\}_0$ are shown in Figures 1, 2, 3, and 4, in which the values of P for each generation are plotted against generation. The



Figs. 1-4.—The simulated effects of selection are shown as mean phenotype of progeny plotted against generation. Figure 1 shows the P set of runs for a population of size 100 progeny/50 parents. Figure 2 shows the replicates of these runs at different starting points in the set of random numbers. Figures 3 and 4 are as for Figures 1 and 2, but for a population of size 40/4. The five selection lines show the effect of different degrees of linkage from A (independence) to E (tight linkage).

first feature of these results is the similarity with experimental data. The programme is, as far as inspection of these "selection lines" can tell, simulating natural systems.

Considering the large populations, i.e. Figures 1 and 2, there is a correlation of rate of advance with tightness of linkage. The effect of linkage is negligible for $r = 0.25$, slight for $r = 0.05$ and 0.025 , and marked for $r = 0.005$. At the latter value there are distinct breaks in the selection lines.

The small populations, i.e. Figures 3 and 4, show, even though there is a much greater intensity of selection, a distinctly slower rate of advance and far greater variability from generation to generation. The effect of linkage is considerably

exaggerated, particularly in the *E* run of Figure 4, in which no advance occurred even after 20 generations of selection.

Comparisons between the replicate runs (Figs. 5 and 6) show fairly close agreement in the large populations, but in one run, the *B* run of Figure 2, the selection line drifted downwards for several generations before regaining its earlier advance. In the small populations, differences between replicates are, as is expected, much greater.

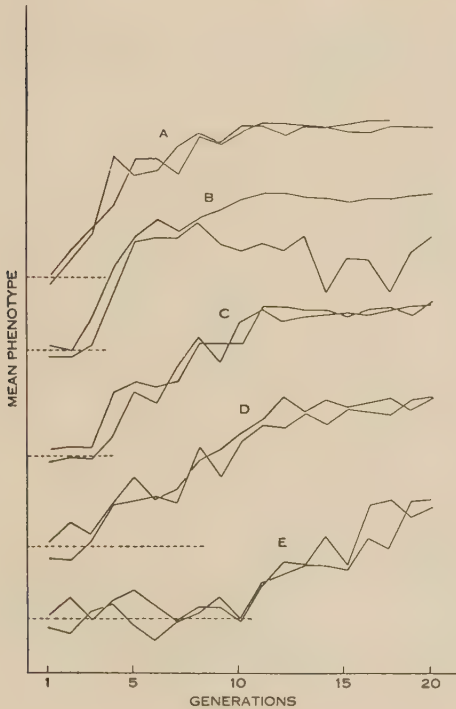


Fig. 5

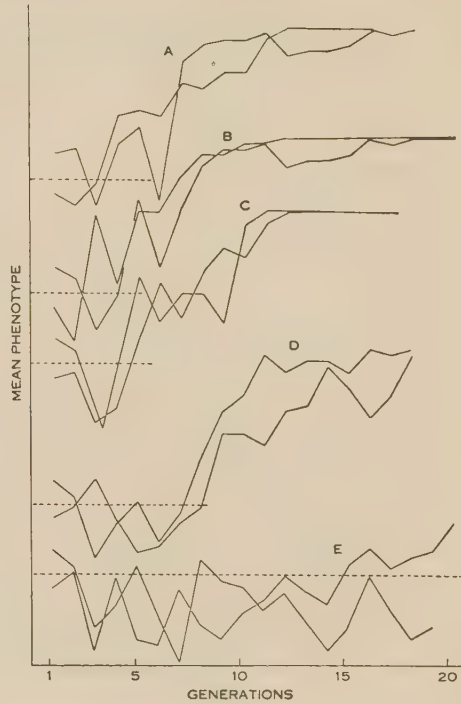


Fig. 6

Figs. 5 and 6.—The replicate runs shown separately in Figures 1–4 are shown plotted together.

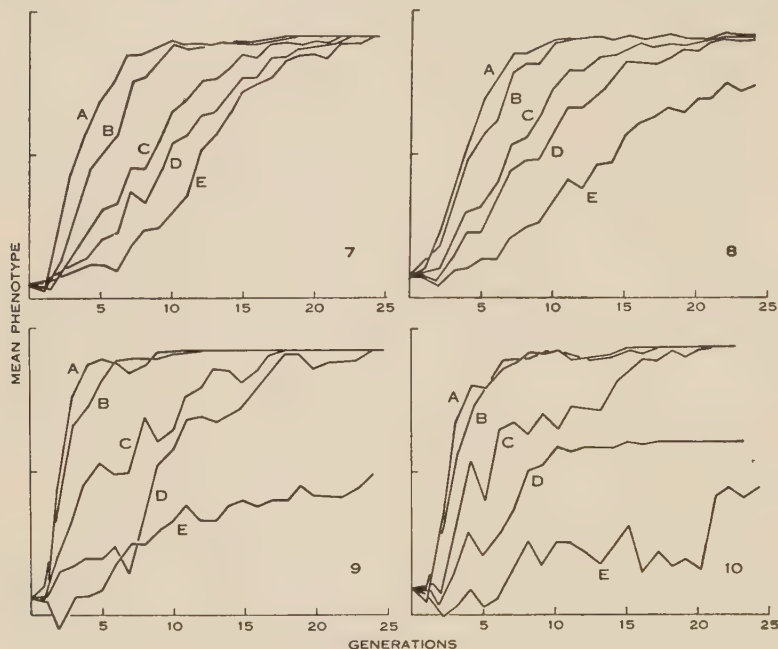
(b) The *Q* Set of Runs

The results of the runs at the *Q* value of $\{g_i\}_0$ are shown in Figures 7, 8, 9, and 10.

The large populations show a clearer relation of the rate of advance under selection to the amount of recombination. Even the *B* runs ($r = 0.25$) show a slower rate of advance, which was not apparent in the *P* set of runs.

The small populations show a markedly different pattern from the large populations. Firstly, there are no differences between the *A* and *B* runs, i.e. reducing recombination from 0.5 to 0.25 has no effect. Secondly, comparing the *A* and *B* runs in the large and small populations, there is a clear difference in the rate of advance; the higher intensity of selection in the small populations is effective in the *Q* set of runs, whereas it was not effective in the *P* set of runs. Thirdly, the effect of the

more intense selection in the small populations is not very marked in the run at $r = 0.05$ (the *C* runs) and there are possible discontinuities in the rate of advance. Fourthly, discontinuities are more marked in the *D* runs and are very clear in the *E* runs. The *E* run of Figure 9 shows two advances separated by a period of 10 generations when no advance occurred. Finally, in the *D* run of Figure 10 fixation of a recessive allele, i.e. an 0 allele, occurred.



Figs. 7-10.—As for Figures 1-4, but for the *Q* set of runs at population sizes of 100/50 (Figs. 7 and 8) and 40/4 (Figs. 9 and 10) respectively.

VI. DISCUSSION

There has been considerable discussion on the importance of linkage in quantitative variation, led by Mather in his construction of the "polygene concept" (cf. Mather 1943). The essence of this concept is that the individual genes determining the variation of a quantitative character occur in linked units, which he terms polygenes. The initial evidence on which he based the concept came from the results of selection experiments. "Selection experiments have shown that such combinations exist in *Drosophila melanogaster* (Mather 1941; Wigan 1942; Sismanidis 1942) and they can be inferred from Winter's (1929) results with maize. The great storage capacity is well brought out by Payne's (1918) selection for increase in the number of scutellar bristles in *Drosophila*. . . . Sismanidis (1942) has confirmed Payne's results and has been able to assign the sudden responses to selection to recombinations in particular chromosomes" (Mather 1943). Sismanidis's results are illustrated in Figure 11, which is after Figure 5 of Mather (1943). The *E* run from Figure 10 is shown in Figure 12 for comparison with Sismanidis's results.

It is clear from the runs of this programme that the detection of the occurrence of linkage in a genetic system from heterogeneity of the rate of advance under selection is unlikely to be possible in large populations. Further where linkage is detectable it is likely to be fairly close, i.e. $r \simeq 0.005$.

The programme used to produce the simulation of the effects of selection suffers from two major defects. These are (1) limitation of the number of loci to less than seven, and (2) lack of provision for other dominance relations than complete dominance. It has, therefore, not been prepared in a form for general use, and a second programme has been written which does not contain these defects. This programme will be discussed in a later paper, and prepared in a form for general use on any ILLIAC-type computer.

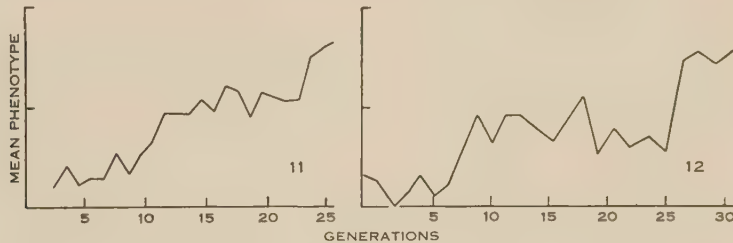


Fig. 11.—Selection for number of chaetae in *Drosophila* (after Mather 1943).

Fig. 12.—The *E* run from Figure 10 shown for comparison with Figure 11.

VII. ACKNOWLEDGMENTS

I am indebted to the staff of the Adolph Basser Computing Laboratory, University of Sydney, particularly Dr. J. O. Bennett, Dr. B. Chartres, and Mr. J. Butcher. Miss J. Ogilvie prepared and checked the many tapes required in this study.

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